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INJECTIONS OF CERTAIN PLANT GROWTH SUBSTANCES IN RATS AND CHICK EMBRYOS

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(Received for publication on May 31, 1943)

A. Introduction

The purpose of the present experiment was to study the effects of certain plant growth substances on animals. Studies were made on growth effects and estrogenic properties of these substances.

It was shown by Boysen-Jensen (1911) and Paal (1919) that by placing the cut tip of the plant stem asymmetrically on the stump the growth of the lower zones was accelerated by a substance coming from the tip. They also demonstrated that this substance was a diffusible agent since it could be transmitted across gelatin. Substances (auxin *a* and *b*) having similar properties as the active principle in the coleoptile tips were isolated and crystallized from urine by Kögl and Haagen-Smit in 1931.

Kögl, *et al* (1934) discovered that indole-acetic acid has the property of promoting growth in stems and illustrates lack of specificity as to chemical structure and related activities in plants. Thimann (1935) showed that indole-acetic acid was produced by microorganisms, as Rhizopus suinis, in a peptone medium rich in tryptophane.

Many compounds have been investigated and are summarized by Zimmerman and Wilcoxon (1935), Zimmerman and Hitchcock (1937), and Went and Thimann (1937). Indole-propionic acid's activity was studied by Hitchcock (1935) and found to have the same ability as indole-3-acetic acid in producing bending, proliferation, and rooting responses in plants. In the case of naphthalene-acetamide and naphthalene-acetic acid, Mitchell and Stewart (1939) found the former was associated with secondary thickening, and the latter with cellular proliferation. Maleic acid, a dicarboxylic acid, was found to be active in inducing renewed cell division and cell extension in certain plants (English, Bonner, and Haagen-Smit, 1939).

In order to study the relationships between plant growth substances and their effects on organisms, many methods of attack have been employed.

Ball (1938) studied the effects of 3-indole-acetic acid on Escherichia coli and found that cell division of the organism was more than doubled in the media containing the chemical as compared with the controls. Continuing these studies on bacteria, Beckwith and Geary (1940) found that indole-3-acetic acid not only increased the growth of Escherichia coli, but also Eberthella typhosa. Working with protozoa (Euglena gracilis), Elliott (1938) observed a marked acceleration of growth using 3-indole-acetic acid, 3-indole-propionic acid and 3-indole-butyric acid. Certain other species were inhibited, however. Du Pan, *et al* (1935) found that hormones of orchid pollinia introduced under the ectoderm of certain Batrachian embryos "during the blastula stage," accelerated cell division.

Recently, Narat and Chobot (1939) have contributed very important observations on the effects of certain plant growth substances on general growth, as well as local cell growth in rats. They used potassium naphthalene acetate and potassium indole butyrate. Growth in rats was not accelerated with injections of either chemical. The effect on local cell growth was pronounced in that both chemicals shortened the healing time of artificially produced burns 35 per cent.

The effects of plant growth substances on tumors have also been investigated. Robinson and Taylor (1941) found that indole-3-acetic acid inhibited normal respiration of both sarcomas and carcinomas in mice, being effective in the undissociated acid form. The effects of potassium alpha-naphthalene acetate on mouse and rat carcinoma were studied by Suminari (1941). He observed inhibition of these carcinomas using this compound. Kögl, Haagen-Smit, and Tonnis (1933) have shown, however, that certain indole compounds occur in carcinomatous tissue in concentrations twice that of normal tissue.

Another interesting aspect of the relation of plant growth substances to animal physiology is the enhancement of the effect of pituitary gonadotropic hormone by certain plant growth hormones as observed by Breneman (1939). He found that the effectiveness of pituitary gonadotropic extract was increased in the immature rat after admixture of indole-butyric acid and alpha-naphthalene-acetic

acid. The action of the plant growth hormone when given separately was questionable.

B. Materials and Methods

The experimental work was divided into three series. The substances tested in each series were as follows: (*a*) Alpha-naphthalene-acetic acid, (*b*) Amide of alpha-naphthalene-acetic acid, (*c*) Beta-indole-acetic acid, (*d*) Beta-indole-propionic acid, (*e*) Maleic acid, and (*f*) Succinic acid.

1. *Series I*

This series consisted of 180 rats, 90 being injected and 90 being controls. Each litter was divided into two groups, one group being injected, the other serving as a control. The animals were weighed and measured at the age of 7 days, and thereafter once a week up to the age of 56 days, then on the 60th day. The time of hair appearance, tooth eruption, eye opening, and vaginal opening were recorded for each rat. Weights were made accurately to 0.1 gm. The rats were measured by vernier calipers, measuring from the tip of the nose to the base of the tail. Variations possible with this method of measuring were noted to be in the range of 3 mm. Tooth eruption was recorded as the time of appearance of an incisor.

Six groups of 15 rats each were injected with each of the substances listed. Each rat was started at 7 days of age on a dosage of 0.5 mgm. (in sesame oil) and injected subcutaneously each day. This dosage was increased until they were receiving 2.0 mgm. daily by four weeks of age, and was given daily until the rats reached 60 days of age. The control rats received sesame oil only. Preliminary work using larger doses of 5.0 and 10.0 mgm. over a period of three weeks or more produced toxic symptoms in the younger rats. Some of the rats died and others showed retardation of growth and patchy distribution of hair.

2. *Series II*

The total number of rats used was 40, 30 being injected and 10 being controls. These rats were all females and approximately two months old. This series was used to test possible estrogenic properties of the six substances listed.

Daily vaginal smears were made on the 40 rats for a period of two

weeks. At the end of the two weeks, 30 of the rats were ovariectomized. A post-operative period of seven days was allowed to elapse before injections were started. Vaginal smears were continued to confirm the expected diestrus smear following ovariectomy.

Six groups of five spayed rats each were injected subcutaneously with 5.0 mgm. daily of each of the six substances (in sesame oil) for three weeks. Daily vaginal smears were made. At the end of the three weeks injection period, all 40 animals were sacrificed and microscopic sections were made of the uterine horn, cervix, and vagina.

3. *Series III*

This series consisted of 480 chick eggs, 240 being injected, and 240 serving as controls. Fertile eggs were obtained from local hatcheries and incubated in an electric incubator at a temperature between 100-105° F. Solutions for injections were prepared by dissolving 1.0 mgm. of each of the substances per 1.0 cc. of phosphate buffer solution (pH 7.4). The resultant pH was 7.0 approximately. These solutions were sterilized by boiling. Injection was carried out on the 10th day of incubation as follows: A hole was made over the air sac by means of a small electric drill, and then with a sterile tuberculin syringe and needle, 0.05 cc. of the solution was injected into the air sac, the hole then being sealed with cellulose tape. Six groups of 40 each were injected on the 10th day of incubation with each of the substances used. Phosphate buffer solution alone was injected into 120 eggs on the same day of incubation. The other 120 eggs were left intact. Weights and observations on development were recorded at hatching time.

C. Results

1. *Series I—Rats*

The average weight, length, and rate of development of the injected rats compared closely to those for the controls (Tables 1, 2, and 3), and showed no significant variations. In regard to the rate of development, there were no significant differences between injected and control animals, except possibly in the opening of the vagina in the animals injected with succinic acid (Table 3). The average age of opening in the injected animals was 57.2 days as compared to 49.4

TABLE 1

THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY WEIGHT OF RATS

Average body wt. in gms. Under each average wt. the range in wts is given. ( ) = No. rats. Injections—daily from 7-60 days of age. Dosage—increased from 0.5 mgm. to 2.0 mgm. during the first 4 weeks of treatment.

Age (days)	Controls	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
7	9.8 (90) 7.0-13.6	10.9 (15) 10.4-11.3	7.2 (15) 7.0- 7.3	11.5 (15) 10.0-12.3	9.0 (15) 7.2-11.2	7.5 (15) 7.0-8.5	10.6 (15) 10.1-11.4
14	19.9 (88) 11.8-25.3	20.6 (14) 20.3-21.4	17.4 (15) 16.9-18.0	22.5 (15) 20.3-23.6	15.9 (15) 11.8-24.0	19.5 (15) 18.0-24.0	20.4 (15) 19.0-22.4
21	25.9 (88) 16-38.5	31.3 (14) 30.5-31.1	27.5 (14) 26.2-29.0	33.1 (15) 30.0-35.5	21.0 (14) 13.0-37.6	32.9 (15) 27.5-36.0	33.2 (15) 30.0-36.4
28	45.5 (86) 32.8-59.6	47.9 (14) 44.2-49.6	40.7 (13) 39.0-44.1	51.9 (15) 47.8-43.6	34.6 (13) 29.3-40.1	45.0 (15) 41.2-47.0	48.1 (15) 45.4-51.0
35	71.7 (86) 45.7-85.3	66.9 (14) 58.4-71.9	59.0 (13) 56.7-62.6	71.8 (15) 66.2-74.1	65.3 (13) 52.5-78.1	60.0 (15) 56.0-65.0	65.2 (15) 63.0-67.0
42	83.4 (86) 66.5-100.0	91.2 (14) 81.3-97.3	73.3 (13) 71.0-75.0	78.7 (15) 72.3-82.3	72.4 (13) 68.1-74.0	77.0 (14) 72.2-83.1	85.2 (14) 68.2-90.1
49	98.3 (86) 79.8-115	108.9 (14) 97.2-115.2	88.8 (13) 85.2-93.1	100.2 (15) 90.9-110.8	89.4 (13) 82-96.4	80.0 (14) 73.4-85.0	99.2 (14) 92.1 106
60	116.5 (86) 90.5-136.3	123.4 (14) 104.8-133.5	112.8 (13) 106.4-116.5	121.3 (15) 111.0-135.0	113.4 (13) 102-119	101.5 (14) 98.4-105	114.1 (14) 102-120

TABLE 2

THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY LENGTH OF RATS

Average body length in mm. Under each average length the range in lengths is given. ( ) = No. rats. Injections—daily from 7-60 days of age. Dosage—increased from 0.5 mgm. to 2.0 mgm. during the first 4 weeks of treatment.

Age (days)	Controls	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
7	60.9 (90) 56.0-70.3	61.0 (15) 60-64	53.0 (15) 52.5-53.3	62.6 (15) 60.0-64.4	61.3 (15) 56-78	58.0 (15) 56-61	59.0 (15) 55.1-65
14	80.0 (88) 66.0-93.0	79.3 (14) 78.3-80	72.2 (15) 70-74	80.7 (15) 78-82	79.2 (15) 58-78	77.8 (15) 72-81	78.2 (15) 76.1-82
21	97.2 (88) 81-109	101.2 (14) 100.5-101.6	93.6 (14) 89-97	101 (15) 94-105	83.0 (14) 68-105	98 (15) 91-106.1	99.1 (15) 92-107
28	119.1 (86) 99-130	125.3 (14) 120-129	112.0 (13) 111-113	118-(15) 113-123	100.0 (13) 90-110	112.0 (15) 107-115	119.0 (15) 105-124
35	136.2 (86) 116-148	130.6 (14) 125-135	125.0 (13) 123-127	131.7 (15) 126-134	127.0 (13) 119-135	120.0 (15) 114-126	128.1 (15) 122-134
42	142.2 (86) 132-153	144.3 (14) 142-146	133.6 (13) 132-135	139.5 (15) 135-143	134.1 (13) 129-141	136.0 (14) 130-141.2	141.0 (14) 133-144
49	149 (86) 139-161	162.3 (14) 144-157	145.3 (13) 145-146	150.2 (15) 146-155	144.2 (13) 139-152	140.0 (14) 133-145	148.1 (14) 141-154
60	160.1 (86) 142-172	165 (14) 153-170	155.0 (13) 150-168	156.7 (15) 146-165	156.0 (13) 144-161	155.0 (14) 150-163	156.0 (14) 151-159

TABLE 3

THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2), BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPRIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE RATE OF DEVELOPMENT OF RATS

( ) = No. of rats. Injections and dosage same as Table 1 and Table 2.

	Hair appeared	Teeth erupted	Eyes opened	Vagina opened
Controls	6 days (90)	9 to 10 days (90)	17 days (90)	49.4 days (av.) 45-56 (range) (40)
Sol. 1	6 days (14)	9 to 10 days (14)	17 days (14)	50.1 days (av.) 46-57 (range) (6)
Sol. 2	6 days (13)	9 to 10 days (13)	17 days (13)	48.4 days (av.) 47-58 (range) (7)
Sol. 3	6 days (13)	9 to 10 days (13)	17 days (13)	54.1 days (av.) 49-58 (range) (7)
Sol. 4	6 days (13)	9 to 10 days (13)	17 days (13)	51.2 days (av.) 48-54 (range) (8)
Sol. 5	6 days (14)	9 to 10 days (14)	17 days (14)	52.0 days (av.) 47-55 (range) (6)
Sol. 6	6 days (14)	9 to 10 days (14)	17 days (14)	57.2 days (av.) 50-61 (range) (4)

for the controls. However, only four animals were used in the injected group.

2. *Series II—Rats*

Daily vaginal smears showed no change from the diestrus smear of ovariectomized rats as compared to the typical 4-day cyclic changes in the vaginal smears of the controls. Microscopic sections of the uterine horn, cervix, and vagina of each of the rats showed no significant changes. In the ovariectomized animals, the vaginal epithelium was atrophic and the walls and lumen were infiltrated with leucocytes. This is in contrast to a control rat killed while in estrus. Here the vaginal epithelium was greatly thickened and cornified. The uterus of the ovariectomized rat was small, anemic, contained atrophied glands, and its wall was infiltrated with leucocytes. The uterus of the rat in estrus was hyperemic, increased in size, and its glands were hypertrophic and hyperplastic.

TABLE 4

THE EFFECT OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 1), AMIDE OF ALPHA-NAPHTHALENE-ACETIC ACID (SOL. 2) BETA-INDOLE-ACETIC ACID (SOL. 3), BETA-INDOLE-PROPIONIC ACID (SOL. 4), MALEIC ACID (SOL. 5), AND SUCCINIC ACID (SOL. 6) ON THE BODY WEIGHT OF CHICK EMBRYOS

Dosage = 0.05 cc. of a 1:1000 solution (in phosphate buffer solution) injected into the air sac on the 10th day of incubation. Average body wt. at hatching in gms. Under each average wt. the range in wts. is given. ( ) = No. of chicks.

Controls	Buffer Sol. only	Sol. 1	Sol. 2	Sol. 3	Sol. 4	Sol. 5	Sol. 6
42.3 (120)	44.3 (120)	40.2 (40)	43.5 (40)	40.9 (40)	43.0 (40)	44.0 (40)	39.5 (40)
38.0-44.2	41.6-47.0	38-41.2	40-46.2	35.5-44	37-44.9	41.2-46	36-41.2

3. *Series III—Chick Eggs*

The average weights of the chicks from the injected eggs compared closely with the controls with the possible exception of the chicks from the eggs injected with solution No. 6—succinic acid (Table 4). Here the average weight of the chicks was 39.5 gms. as compared with 42.3 gms. for the intact controls, and 44.3 gms. for the controls injected with prosphate buffer solution alone. No abnormalities of development were noted at hatching time. One-fourth of the chicks were saved, and all developed without incident to maturity.

D. Conclusions

Alpha-naphthalene-acetic acid, amide of alpha-naphthalene-acetic acid, beta-indole-acetic acid, beta-indole-propionic acid, maleic acid, and succinic acid produced no significant effects on growth and rate of development in albino rats and chick embryos, nor did they exhibit any estrogenic properties in the doses used.

E. Summary

1. The effects of six plant growth substances on growth and development were studied in 180 albino rats. Ninety of the rats were injected (6 groups of 15 each) with the substances. The other 90 served as controls.

2. The rats were injected with a maximum of 2.0 mg. daily of each of the substances, and all were weighed and measured each week. No significant differences in the rate of growth and development between the injected and control animals were found.

3. Injections of 5.0 mgm. daily for three weeks into 30 ovariectomized rats did not change their typical diestrus vaginal smear.

4. Injections of 0.05 cc. of a 1:1000 solution of each of the six substances were made into 240 chick eggs on the 10th day of incubation. Controls consisted of 120 intact eggs and 120 eggs injected with phosphate buffer solution. No significant differences in weight or rate of development between the chicks of the injected and control eggs were noted at hatching.

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THE EFFECT OF TUMOR EXTRACTS ON THE GROWTH OF CELLS IN VITRO*[1]

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It has been generally accepted that extracts of tumors possess a marked capacity to stimulate the growth of cells in vitro and that in this respect they resemble proliferating tissue in general (embryonic tissue, bone marrow, etc.) as opposed to mitotically inactive tissues of adult animals.

Investigations carried out in recent years (26, 8, 17, 19) have disclosed facts which give a different aspect to this question. It has been shown, in confirmation of early and completely overlooked experiments of Carrel (3, 6) and Walton (27) that the ability to activate the growth of cell colonies in vitro is not a specific property of extracts of embryonic and proliferating tissues. Extracts of stable adult tissues were found to stimulate cell proliferation to a very marked degree.

The stimulating property is not confined to definite organs. Extracts of all adult tissues and organs possess, though in varying degree, the ability to activate cell growth. The growth promoting activity of extracts of different organs is not correlated with the state of proliferation of the organ or tissue from which the extract is made. Extracts of bone marrow, for instance, possess only moderate growth stimulating activity, whereas extracts of mitotically stable organs (such as brain and heart) are extremely active. Under given experimental conditions extracts of heart muscle stimulate the growth of cell colonies about three times as much as embryonic extract in equivalent concentration.

The cell growth activating effect of extracts of adult organs is not transitory: it is possible to cultivate fibroblast colonies indefinitely in media solely composed of adult plasma and extracts of adult tissues (9). The action of extracts of adult tissue is not species-speci-

*A preliminary report has been published (20).

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fic. The growth of chicken fibroblast cultures is stimulated as well, or almost as well, by extracts of organs of heterologous species (cow, sheep, rabbit, dog) as by extracts of chicken tissues (10). In this connection it is necessary to mention that rat (16) and hamster tissues[2] act differently than those of most other animals with regard to cell growth activation. In both rat and hamster extracts of all organs and tissues except brain and embryonic tissues are devoid or almost devoid of cell growth promoting properties and often inhibit cell growth in vitro.

In the light of these findings it seemed desirable to re-examine previous work on the growth stimulating properties of tumor extracts and to make a systematic comparison of the growth promoting effects of extracts of tumors with those of extracts of normal adult tissues.

Material and Procedure

1. *Experimental Procedure*

The experiments were carried out with extracts of the following tumors: (*a*) Rous sarcoma of fowl; (*b*) lymphosarcoma of dog;[3] (*c*) benzpyrene sarcoma of rat (7); (*d*) benzpyrene sarcoma of hamster (15).

The tumor material was selected so that in two cases (chicken and dog) the tumors employed were derived from animals whose normal tissue extracts greatly stimulate cell growth, and in two cases (rat and hamster) from animals whose normal tissue extracts do not appreciably affect cell proliferation or even inhibit it.

The growth of cultures in media containing tumor extracts was compared with the growth of control cultures (sister halves) in (*a*) protective medium composed of plasma diluted with Tyrode solution in the proportion 1:2, and (*b*) in media containing extract of normal adult tissue from the species under trial. Extract of heart muscle of normal adult animals served in each case for the comparison.

2. *Preparation of Tumor Extracts and Heart Extracts*

Tumors in the 14th-30th day of their development and in various

[2]Unpublished experiments.
[3]We are greatly indebted to Dr. B. Gellei for supplying us with this material.

states of proliferation were employed. Care was taken to use only tumor parts which were free from necrosis.

Hearts for extract preparation were removed from normal adult animals, freed of fat, and then used in their entirety.

Both the tumor material and heart muscle were minced finely with scissors, suspended in five parts of Tyrode solution, then left to stand for 24 hrs. in the refrigerator. The suspension was centrifuged for 5 min. and the supernatant fluid decanted off. Extract thus obtained was stored in the refrigerator. It remains in good condition for weeks.

3. *Tissue Culture Technique*

The stimulating effect of tumor extract was tested on chicken fibroblast colonies growing in Carrel flasks. Flask cultures were prepared as follows: Standardized cover-slip cultures of fibroblasts were divided into two equal parts and transferred to flasks: one half was used as a control, the other served for experiment. The solid phase of the medium in which the cultures were planted was composed of chicken plasma diluted with Tyrode solution in a proportion of 1:2. Tumor extract and heart muscle extract respectively were added as supernatant fluid phase. The latter was not changed throughout the entire course of the experiment. The course of growth was followed for a period of 6-8 days. Growth curves were constructed by planimetric measurements of the surface area of the cultures according to the method of Ebeling.

Results

1. *Effect of Extract of Rous Chicken Sarcoma on the Growth of Fibroblasts in Vitro*

Extracts of Rous sarcoma exert a marked growth promoting effect on fibroblast cultures (Figure 1*A*). Cultures growing in protective medium after six days reached a mean size of 13.9 mm^2, whereas sister halves cultivated in medium containing Rous sarcoma extract for the same time reached a mean size of 40.8 mm^2 (Table 1).

The growth promoting effects of extract of Rous sarcoma and of adult chicken heart are compared in Table 2. Only very seldom does the activity of Rous sarcoma extract exceed that of heart extract. In most cases it is somewhat less (Figure 1*B*). A comparison of the value of the mean size of the cultures in medium containing Rous sarcoma

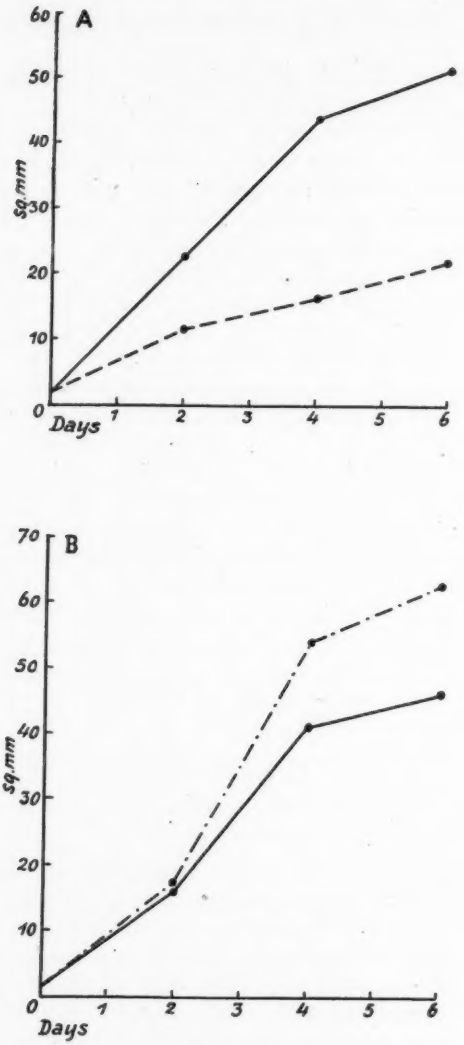

FIGURE 1

GROWTH CURVES OF SISTER HALVES OF FIBROBLAST CULTURES

A: in protective medium and in medium containing Rous sarcoma extract (Experiment No. 7908);

B: in medium containing fowl heart extract and in medium containing Rous sarcoma extract (Experiment No. 787b).

———— Rous sarcoma extract;

-.-.-.-.-.-.- fowl heart extract;

- - - - - - - - - - protective medium.

TABLE 1

Culture No.	Size of cultures in mm² grown 6 days in: Medium containing Rous sarcoma extract	Protective medium
790*a*	48	19
790*b*	56	18
791*a*	40	18
791*b*	22	9
798*a*	24	10
802*a*	31	7
803*b*	25	13
833*a*	47	15
833*b*	32	7
834*a*	27	13
834*b*	30	13
837*a*	30	8
837*b*	21	12
839*a*	40	14
839*b*	35	16
840*a*	95	15
840*b*	63	29
880*a*	42	17
880*b*	42	19
881*a*	43	17
881*b*	37	10
882*a*	32	7
882*b*	48	10
887*a*	57	18
887*b*	54	13
888*a*	53	13
888*b*	36	13
889*a*	33	18
Mean	41	14

extract with that of cultures in heart extract medium shows that the activity of Rous sarcoma extract is about 81 per cent that of heart extract.

The appearance of cultures growing in media containing Rous sarcoma extract is completely normal. The colonies are regular in architecture and healthy in appearance. The individual cells may be either quite transparent and clear or somewhat granular.

The growth promoting activity of extracts of Rous sarcoma was tested on 28 pairs of cultures and the comparison of the action of Rous sarcoma extract with that of extract of chicken heart was carried out on 62 pairs of cultures. In all, extracts of 10 different tumors were tested.

TABLE 2

Culture No.	Size of cultures in mm² grown 6 days in medium containing Rous sarcoma extract	Heart extract
787*a*	60	79
787*b*	45	60
788*a*	32	49
788*b*	51	58
789*a*	35	47
789*b*	46	66
835*a*	26	35
835*b*	45	65
836*a*	38	41
836*b*	40	33
843*a*	55	43
843*b*	31	38
844*a*	40	39
844*b*	44	49
851*a*	30	27
851*b*	25	43
852*a*	31	49
852*b*	55	53
860*a*	20	65
860*b*	13	60
861*a*	33	58
861*b*	50	53
863*a*	28	50
863*b*	26	38
865*a*	60	60
865*b*	53	68
866*a*	79	72
866*b*	91	87
867*a*	58	67
867*b*	65	62
868*a*	88	67
868*b*	63	82
869*b*	17	60
870*a*	19	68
870*b*	30	48
871*a*	72	88
871*b*	57	92
872*a*	82	93
872*b*	70	70
873*a*	32	85
874*a*	67	56
874*b*	83	66
875*a*	55	55
875*b*	49	83
877*a*	118	98
877*b*	88	90
878*a*	64	87
878*b*	94	94
883*a*	65	70

TABLE 2 (Continued)

Culture No.	Size of cultures in mm² grown 6 days in medium containing Rous sarcoma extract	Heart extract
883*b*	56	47
884*a*	62	55
884*b*	35	63
885*a*	46	58
885*b*	25	37
892*b*	27	45
894*a*	32	31
897*a*	39	51
897*b*	38	57
898*a*	15	28
898*b*	39	43
899*a*	33	41
899*b*	19	38
Mean	48	58

2. *The Effect of Extract of Dog Lymphosarcoma on the Growth of Fibroblasts in Vitro*

Table 3 gives the size of cultures growing in media containing tumor extract and in protective medium respectively. Extracts of lympho-

TABLE 3

No.	Size of cultures in mm² grown 6 days in Medium containing dog tumor extract	Protective medium
5*a*	48	7
5*b*	46	9
31*a*	33	8
31*b*	34	16
33*a*	38	4
33*b*	49	12
35*a*	51	12
35*b*	88	20
Mean	48	11

sarcoma of dog definitely stimulate the growth of fibroblast colonies in vitro (Figure 2*A*).

A comparison of the growth promoting effect of extracts of dog lymphosarcoma with that of adult dog heart shows that the tumor extracts are somewhat more active (Figure 2*B*). Cultures grown in

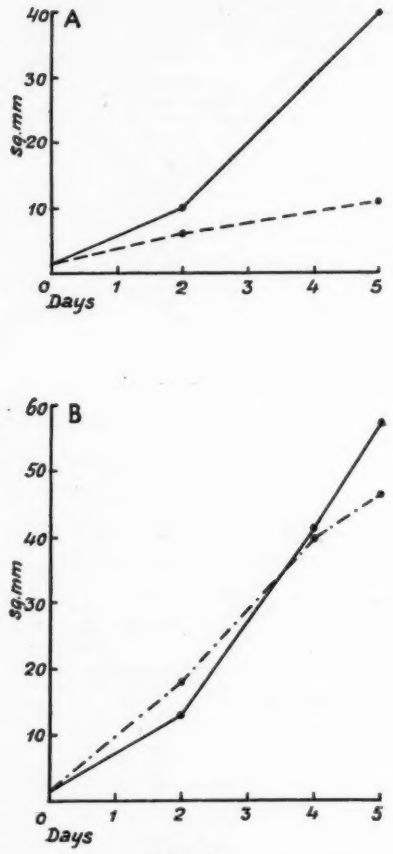

FIGURE 2

GROWTH CURVES OF SISTER HALVES OF FIBROBLAST CULTURES

A: in protective medium and in medium containing dog lymphosarcoma extract (Experiment No. 34a);

B: in medium containing dog heart extract and in medium containing dog lymphosarcoma extract (Experiment No. 8a).

———— Dog lymphosarcoma extract;

-.-.-.-.-.-.- dog heart extract;

- - - - - - - - - - - protective medium.

tumor extract medium are about 25 per cent larger than cultures grown in heart muscle extract medium (Table 4).

TABLE 4

Culture No.	Size of cultures in mm² grown 6 days in medium containing Dog tumor extract	Dog heart extract
9*a*	47	46
9*b*	70	64
25*a*	50	25
25*b*	33	22
27*a*	30	17
27*b*	27	16
29*a*	40	24
29*b*	33	25
39*a*	28	39
41*a*	64	39
41*b*	58	37
Mean	44	32

The appearance of the cells is healthy and the structure of the colonies is uniform and similar in both cases.

The growth promoting activity of extracts of dog lymphosarcoma was tested on eight pairs of cultures. The comparison of the activity of dog tumor extracts with that of dog heart extract was made on 11 pairs of cultures. Extracts from three different tumors were tested.

3. *The Effect of Extracts of Rat Benzpyrene Sarcoma on the Growth of Fibroblast Cultures in Vitro*

Extracts of rat benzpyrene sarcoma definitely inhibit the growth of fibroblast cultures in vitro (Figure 3*A*). As may be seen from Table 5, cultures growing in medium containing rat sarcoma extract reach in six days a mean size of 7.8mm² whereas cultures growing in protective medium reach in the same period a mean size of 17.7mm.²

A comparison of the size of cultures growing in media containing rat heart extract with that of cultures growing in media containing rat tumor extract shows that cultures grow much more slowly in the latter. Rat tumor extract markedly inhibits the growth of fibroblasts in vitro whereas rat heart extract has only a very slight inhibitory effect or no effect at all on cell outgrowth (Table 6, Figure 3*B*).

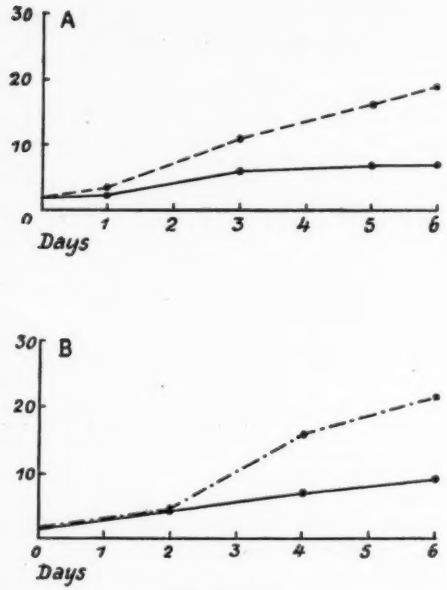

FIGURE 3

GROWTH CURVES OF SISTER HALVES OF FIBROBLAST CULTURES

A: in protective medium and in medium containing rat sarcoma extract (Experiment No. 49a);

B: in medium containing rat heart extract and in medium containing rat sarcoma extract (Experiment No. 13).

———— Rat sarcoma extract;
–.–.–.–.–.–.– rat heart extract;
– – – – – – – – – – protective medium.

Although cultures growing in medium containing rat sarcoma extract are small, they are uniform in structure and the cells are quite normal in appearance. There is no difference in appearance between cultures grown in heart extract and in tumor extract medium.

The activity of rat sarcoma extract was tested on 14 pairs of cultures. The comparison of the activity of rat tumor extracts with that of rat heart extract was made on 15 pairs of cultures. In all, extracts from six different tumors were tested.

TABLE 5

Culture No.	Size of cultures in mm² grown 6 days in Medium containing rat tumor extract	Protective medium
7*a*	8	7
7*b*	4	9
13*a*	12	18
13*b*	8	17
14*a*	10	10
14*b*	7	16
17*a*	4	29
17*b*	4	17
47*a*	12	22
47*b*	9	19
48*a*	6	17
48*b*	10	32
49*a*	7	16
49*b*	8	20
Mean	8	18

TABLE 6

Culture No.	Size of cultures in mm² grown 6 days in medium containing Rat tumor extract	Rat heart extract
13*a*	9	35
13*b*	9	24
57*a*	6	6
57*b*	7	10
59*a*	5	7
59*b*	5	5
69*a*	5	11
69*b*	5	13
71*a*	14	18
71*b*	9	13
72*a*	18	13
73*a*	7	10
73*b*	6	7
75*a*	11	15
75*b*	5	15
Mean	8	14

4. *The Effect of Extracts of Hamster Benzpyrene Sarcoma on the Growth of Fibroblast Cultures in Vitro*

As Table 7 shows, extracts of hamster tumors have a marked inhibiting effect on the growth of fibroblasts in vitro. Cultures growing

in medium containing hamster tumor extracts are smaller than those growing in protective medium (Figure 4*A*). A comparison of cultures

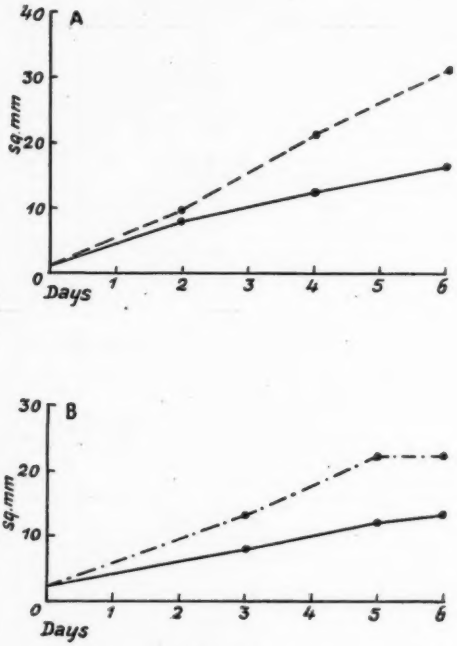

FIGURE 4

GROWTH CURVES OF SISTER HALVES OF FIBROBLAST CULTURES

A: in protective medium and in medium containing hamster sarcoma extract (Experiment No. 107/8);

B: in medium containing hamster heart extract and in medium containing hamster sarcoma extract (Experiment No. 61/2b).

———— Hamster sarcoma extract;
-.-.-.-.-.-.- hamster heart extract;
---------- protective medium.

growing in media containing hamster heart extract with cultures growing in media containing hamster tumor extracts shows that cultures grow more slowly in the latter (Figure 4*B*). Hamster tumor extract markedly inhibits the growth of fibroblasts in vitro, whereas hamster

TABLE 7

Culture No.	Size of cultures in mm² grown 6 days in Medium containing hamster tumor extract	Protective medium
1*a*	15	18
1*b*	18	14
13*a*	12	6
13*b*	7	5
39*b*	9	7
40*a*	4	6
40*b*	2	6
46*a*	18	46
46*b*	20	26
67*a*	8	17
67*b*	12	19
68*a*	10	11
68*b*	15	14
101*a*	19	23
101*b*	26	31
103*a*	13	16
103*b*	10	22
105*a*	22	34
105*b*	13	41
107*a*	16	31
107*b*	13	32
Mean	13	20

TABLE 8

Culture No.	Size of cultures in mm² grown 6 days in medium containing Hamster tumor extract	Hamster heart extract
42*a*	18	12
43*a*	7	18
43*b*	11	20
44*a*	11	10
44*b*	6	10
45*a*	16	23
45*b*	23	27
61*a*	15	48
61*b*	13	23
93*a*	10	24
93*b*	3	15
95*a*	12	23
95*b*	14	18
97*a*	9	26
97*b*	11	19
99*a*	6	10
99*b*	7	21
Mean	11	20

heart extracts have either no effect at all or stimulate cell outgrowth only very slightly (Table 8).

The activity of hamster sarcoma extract was tested on 21 pairs of cultures. The comparison of the activity of hamster tumor extract with that of hamster heart extract was carried out on 17 pairs of cultures. A total of six hamster tumors was tested.

Summary

The results of foregoing experiments may be summarized as follows:

1. Extract of chicken Rous sarcoma stimulates the growth of cultures of chicken fibroblasts in vitro to a marked degree. The growth promoting activity of Rous sarcoma extracts is somewhat weaker than that of extracts of heart muscle of adult chicken.
2. Extracts of dog lymphosarcoma have pronounced growth stimulating properties. Their activity is somewhat greater than that of extracts of adult dog heart.
3. Extracts of rat sarcoma have a pronounced growth inhibiting effect on the outgrowth of fibroblasts in vitro.
4. Extracts of hamster sarcoma inhibit the growth of fibroblasts in vitro.

Discussion

Carrel and Burrows (5) were the first to describe the growth activating effect of tumor extracts on cell cultures in vitro. They cultivated adult and embryonic spleen in chicken plasma to which Rous sarcoma extract was added. Under these conditions good growth of cells was observed and sometimes an enormous acceleration of cell proliferation took place. A comparison of the growth promoting activity of chicken sarcoma extract with that of embryonic extract showed that tumor extract has a more energetic stimulating action. In a later paper Carrel (3) confirmed his previous finding concerning the growth stimulating property of Rous sarcoma extract but claimed that its activity is somewhat lower than that of embryonic extract. Lambert (22) questioned these findings and stated that in any case it is certain that they do not possess any general application. He found that an extract of human tumor employed by him appeared to inhibit rather than stimulate the growth of normal human cells. The fact that the activity of tumor extract is not uniform was later recognized

by Carrel (4). He compared the growth activating effect of extracts of various chicken sarcomas and found that the activity of the extract varies with the tumor. "En général la présence de l'extrait produisit une augmentation marquée de la prolifération cellulaire pendant quelques jours. . . . Mais souvent, l'activation de la croissance ne dura que quelque jours. . . . Dans plusieurs cas le suc de sarcome ne détermina d'emblée aucune accélération de la croissance et amena une mort rapide des fibroblastes."

The problem of the growth stimulating action of tumor extracts was later taken up by a number of other workers. Drew (11, 12) found that extracts of various tumors (mouse carcinoma 37, mouse carcinoma 63, rat sarcoma JRS) possess the power of producing active proliferation of tissues in vitro. These extracts "were often found to be very erratic, different extracts of the same strain of tumor but prepared at various times, sometimes giving a powerful extract and in others being inert. The watery extracts from transplantable tumors varied in activity according as they were prepared from tumors growing rapidly or from receding tumors."

Biceglie (1, 2) stated that tumor extracts greatly stimulate the growth of some tissues such as spleen and thyroid, whereas they inhibit the growth of others.

Takeguchi (25) found that extracts of carcinoma of cervix uteri inhibit the growth of chicken fibroblasts. The inhibition was not as marked when extracts of a healthy human uterine cervix were used.

Mottram (24) reported that extracts of Jensen rat sarcoma activated the growth of kidney cultures.

Earle (13) stated that extracts of Walker rat mammary carcinoma No. 256 tremendously stimulate the growth of tumor epithelium but not of fibroblasts, and that tumor extracts have no growth promoting action on normal mammary epithelium.

A comparison of the results of our experiments with those of the authors cited above is difficult. Most of the investigations mentioned were carried out before tissue culture technique was developed to a quantitative method and the more recent studies were made without advantage being taken of the possibilities presented by the improved technique. It can only be stated that the early observations of Carrel and Burrows and of Drew on the growth promoting activity of tumor extracts could be corroborated by us for certain kinds of tumors.

The most important point to be concluded from our work is that extracts of tumors affect cell growth in vitro in essentially the same manner as extracts of normal tissues (heart muscle).

The influence of tumor extracts on the growth of cultures in vitro is by no means uniform. Extracts of some tumors stimulate cell growth and extracts of others inhibit it depending upon the species from which the tumor is derived. Of the kinds of tumors which were tested two were derived from animals (chicken and dog) whose normal tissue extracts markedly stimulate cell growth in vitro, and two from animals (rat and hamster) whose normal tissue extracts do not appreciably affect cell proliferation or even inhibit it. The action of extracts of both groups of tumors showed corresponding differences in activity. The tumors of the first group markedly stimulated cell growth in vitro, whereas those of the second group inhibited it.

Emphasis should be laid on the fact that the growth promoting power of those tumor extracts which stimulate cell growth is never exceptionally high. It falls in the range of activity manifested by extracts of heart muscle. Extracts of Rous chicken sarcoma are somewhat less active, while dog lymphosarcoma extracts are somewhat more active than the corresponding heart extracts.

In considering the etiology of tumors the question has often been asked whether neoplastic growth can be attributed to an unusually enhanced growth capacity of malignant cells or to the presence of an unusually large amount of growth promoting factors in cancer tissue. Recent investigations carried out on malignant cells grown in vitro have clearly shown that under the experimental conditions of life in vitro cancer cells do not possess a greater growth capacity than embryonic cells (18). Malignant cells are not different in this respect from normal cells of the adult organism which also grow vigorously in vitro (24). The investigations reported in this paper make it clear that it is likewise impossible to speak of the presence in neoplastic tissues of an unusual amount of growth promoting factors.

If one accepts the hypothesis that malignancy is related to a break in the "balanced control" to which the normal cells are subjected in vivo (Murphy, 23) one should, in the light of these investigations, not attribute the disturbance in balance to an increase in growth capacity or growth stimulating factors. Experiments seem much more to justify the idea that the local conditions which lead to tumor forma-

tion are not such as primarily favour growth but rather such as lead to a break in the system of forces which control and limit cell proliferation in the organism.

Summary

The action of extracts of four kinds of tumors (Rous sarcoma of fowl; lymphosarcoma of dog; benzpyrene sarcoma of rat; benzpyrene sarcoma of hamster) on the growth of fibroblast colonies in vitro was examined. The tumor material was selected so that in two cases (chicken and dog) the tumors employed were derived from animals whose normal tissue extracts greatly stimulate cell growth, and in two cases (rat and hamster) from animals whose normal tissue extracts do not appreciably affect cell proliferation or even inhibit it. The growth of cultures in media containing tumor extracts was compared with the growth of controls (*a*) in protective medium and (*b*) in medium containing extract of normal adult tissue (heart muscle) from the same animal. The results of the experiments may be summarized as follows.

1. Extract of chicken Rous sarcoma stimulates the growth of cultures of chicken fibroblasts in vitro to a marked degree. The growth promoting activity of Rous sarcoma extracts is somewhat weaker than that of extracts of heart muscle of adult chicken.

2. Extracts of dog lymphosarcoma have pronounced growth stimulating properties. Their activity is somewhat greater than that of extracts of adult dog heart.

3. Extracts of rat sarcoma have a pronounced growth inhibiting effect on the outgrowth of fibroblasts in vitro.

4. Extracts of hamster sarcoma inhibit the growth of fibroblasts in vitro.

From the above experiments the conclusion has been drawn that extracts of tumors affect the rate of cell growth in vitro in essentially the same manner as extracts of normal tissues. In the cases where normal tissue extract stimulated cell growth, tumor extracts acted likewise; in the cases where extracts of normal tissues either did not appreciably affect cell proliferation or inhibited it, tumor extracts inhibited the growth of cultures. The growth promoting power of those tumor extracts which stimulate cell growth is never exceptionally high; it falls in the range of activity of extracts of adult heart.

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ONTOGENETIC SIZE DIFFERENTIATION IN SINGLE COMB WHITE LEGHORNS

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In a previous communication (Lerner, 1943) establishment of two lines of Single Comb White Leghorn chickens differing in body size was described. The criterion of size used was the mature shank length of females which in the original production-bred flock averaged 9.69 cm. Through selection and progeny testing a "Size" line was created with respective shank lengths of 9.92, 9.94, 10.20, and 10.29 cm. in four successive generations. The "Production" line not selected for size varied in the corresponding years from 9.37 to 9.65 cm. in shank length.

There are three possible paths by which the size difference between the two lines may have arisen in the course of development: (*a*) the original zygotes were different in size, (*b*) the rate of growth of the two lines differed, and (*c*) the duration of growth was not the same. In studies of size differentiation in embryos of different avian species all three of these conditions were found singly and in combination by several investigators (Kaufman, 1930; Henderson and Penquite, 1934; Schmalhausen in Kaplansky *et al.*, 1935). Their data, however, deal exclusively with embryonic weight obtained either directly or by computation from surface measurements. Similar studies of intraspecific variation in chickens, where the differences in duration of embryonic growth are either non-existent or negligible, led to contradictory interpretations of results with respect to the effect of differences in rate of growth (Blunn and Gregory, 1935; Byerly, Helsel, and Quinn, 1938). In the present material shank length was the primary criterion of size, and though it is correlated with body weight (Lerner, 1937), certain differences in the characteristics of growth of the two variables would preclude the possibility of generalization from one to the other. Differences in growth pattern of shank length and body weight (Lerner, 1938) and in the duration of their growth (Jaap,

1941) may be cited in this connection. Hence an investigation of the actual material on hand is necessary to establish the ontogenetic basis of the size differences in the lines of birds under question.

Material and Methods

The material used for this study consisted of one hatch (out of a total of five) of the fifth selected generation of Size-line pullets and of Production-line birds hatched at the same time in the same incubators and reared together. These birds were also used in a nutrition experiment in the comparison of two diets, which differed particularly in amount and kind of protein concentrates. The details of the latter experiment are irrelevant for the purpose of this paper. Suffice it to say that the difference in the rations (designated *A* and *B* here) affected the rate of increase in body weight, so that the birds raised on Ration *B* outweighed those on Ration *A* in the early stages of growth. Thus in the analysis reported below it was necessary to consider the variance in growth due to nutrition as well as that due to heredity. At the time of hatching the chicks were segregated on the basis of odd and even wingbands into the two lots (*A* and *B*). In such manner approximately equal numbers of birds of like origin were placed on each of the diets. The shank length of all females was measured as previously described (Burmester and Lerner, 1937) at four weeks of age and every four weeks thereafter till 28 weeks of age. Only females which survived up to the latter age were included in this analysis.

Absolute Length of Shank

Table 1 presents the mean shank length at the different ages for the four subgroups (2 rations × 2 lines). Comparing the Size and

TABLE 1

Mean Shank Lengths in cm.

Line	Ration	Number	Age in weeks 4	8	12	16	20	24	28
Size	*A*	20	3.97	5.99	7.80	9.54	10.38	10.53	10.57
	B	26	4.17	6.12	8.14	9.83	10.42	10.50	10.52
Production	*A*	69	3.92	5.84	7.51	8.88	9.40	9.50	9.53
	B	74	4.06	5.97	7.68	9.04	9.41	9.48	9.51

the Production lines on the same rations it may be seen that at the earliest recorded age some difference in shank length is already established. This difference tended to increase with increasing age. Comparison within lines similarly indicates an inter-ration difference at four weeks of age which in absolute terms tended to increase till 12 weeks of age, following which a decrease is observed. By 20 weeks of age the birds on Ration *A* overcame their original disadvantage and their shanks were longer than of the birds on Ration *B*, but the differential is not very large.

The statistical significance of these differences may be evaluated from the partitioned variance presented in Table 2 as well as from the percentage distribution of the variance appearing in Table 3.

TABLE 2
SHANK-LENGTH VARIANCE

Row	Source of variance	Degrees of freedom	Age in weeks 4	8	12	16	20	24
1	Total	188	0.096	0.343	0.599	0.417	0.338	0.339
2	Between rations	1	1.23	0.89	2.36	2.23	0.15	0.01
3	Within rations	187	0.090	0.340	0.590	0.407	0.339	0.340
4	Pooled estimate, between lines	2	0.135	0.38	2.235	9.575	17.22	18.19
5	within lines	185	0.089	0.340	0.567	0.308	0.156	0.147
6	between sires	26	0.122	0.508	0.689	0.590	0.399	0.413
7	within sires	159	0.084	0.312	0.547	0.262	0.116	0.104
8	Ration *A*, total	88	0.074	0.307	0.700	0.460	0.367	0.367
9	between lines	1	0.05	0.34	1.28	6.85	14.82	16.36
10	within lines	87	0.075	0.307	0.693	0.386	0.201	0.183
11	Size line, total	19	0.086	0.401	0.681	0.401	0.120	0.118
12	between sires	2	0.365	1.495	1.855	0.745	0.12	0.17
13	within sires	17	0.054	0.272	0.542	0.360	0.120	0.112
14	Production line, total	68	0.071	0.280	0.696	0.382	0.223	0.202
15	between sires	11	0.094	0.455	0.991	0.968	0.703	0.671
16	within sires	57	0.067	0.246	0.639	0.269	0.131	0.111
17	Ration *B*, total	99	0.104	0.370	0.493	0.360	0.314	0.316
18	between lines	1	0.22	0.42	4.19	12.30	19.62	20.02
19	within lines	98	0.102	0.369	0.455	0.238	0.117	0.115
20	Size line, total	25	0.138	0.539	0.691	0.415	0.153	0.149
21	between sires	2	0.07	0.135	0.05	0.135	0.12	0.11
22	within sires	23	0.143	0.574	0.747	0.440	0.156	0.153
23	Production line, total	73	0.090	0.312	0.374	0.177	0.104	0.104
24	between sires	11	0.116	0.449	0.291	0.267	0.196	0.255
25	within sires	62	0.086	0.287	0.389	0.161	0.088	0.077

With reference to the first of these tables it is clear from Rows 2, 3, and 7 that the difference of highest significance between rations occurs at four weeks of age. Between 4 and 16 weeks of age the *P* point

TABLE 3
DISTRIBUTION OF SOURCES OF VARIANCE OF SHANK LENGTH

Row	Source	Percentage of variance at different ages					
		4	8	12	16	20	24
1	Total	100.0	100.0	100.0	100.0	100.0	100.0
2	Rations	6.3	0.9	1.5	2.4	0*	0*
3	Lines	1.0	0	3.8	23.7	53.8	56.6
4	Sires within lines	5.2	8.2	3.3	11.0	11.8	12.7
5	Residual	87.5	90.9	91.4	62.9	34.4	30.7
6	Lines within Ration *A*	0*	0	1.0	16.1	45.2	50.1
7	within Ration *B*	1.9	0.3	8.4	33.9	62.7	63.6
	Sires within size line,						
8	Ration *A*	37.2	32.1	20.4	10.2	0	5.1
9	Ration *B*	0*	0*	0*	0*	0*	0*
	Sires within production line,						
10	Ration *A*	5.6	12.1	8.2	29.6	41.3	45.0
11	Ration *B*	4.4	8.0	0*	9.0	15.4	26.0

*Error within class greater than total error.

fluctuates between >0.05 and <0.01, while at 20 and 24 weeks the effect of the ration is no longer in evidence. The variances at 28 weeks of age were not computed, since it is obvious that their distribution is the same as at 20 and 24 weeks of age. In fact the 24-week variance can be considered to be identical with that at 20 weeks within the limits of sampling errors.

The effect of lines (Rows 4-7) in contrast with that of rations is not significant until 12 weeks of age, after which the differences between lines become increasingly significant as the *P* value showed a precipitous drop. Table 3 shows that whereas the percentage of the total variance attributable to ration differences drops from 6.3 at four weeks of age to 0 at 20 and 24 weeks, that due to differences between lines increases from 1.0 per cent to 56.6 per cent during the same period.

Rows 8-25 of Table 2 present a further breakdown of the observed variance within the two rations. Comparison of Rows 9-10 with Rows 18-19 indicates that Ration *B* (on which longer shanks in the early stages were observed) provides a greater differentiation between lines than does Ration *A*. However, the variation between the offspring of different sires within the lines (Rows 12-13 *vs.* 21-22, and Rows 15-16 vs. 24-25) was relatively greater on Ration *A* than on Ration *B*. Since the variance between lines and the variance between sires within lines are both parts of the total genetic variability it is not surprising that increase in one is accompanied by a decrease in the other. It thus seems that the ration which promotes more rapid

growth (Ration *B*) emphasizes the inter-line variance at the expense of the genetic intra-line variance. A ration of this type may then be useful in the selection of major growth differentials, such as distinguish the Size and the Production lines, but would retard selection progress within relatively homogeneous groups, such as represented by the two lines.

This is also brought out by the figures in Table 3. The percentage of the variance due to differences between lines is greater on Ration *B* than on Ration *A* at every age (Rows 6 and 7). That due to differences between sires, however, is uniformly greater on Ration *A* (Row 8 *vs.* 9, and Row 10 *vs.* 11). It should be noted, of course, that the differences credited here to sires also include those due to the differences between groups of dams mated to the different sires.

To summarize this section it may then be said that the differences in shank length between the lines studied are slight up to 12 weeks of age but become increasingly significant with age. The differentiation appears to approach the maximum at 20 weeks of age with only a very slight further increase. The effect of the ration, on the other hand, is at its maximum at the earliest age measured and, although persisting to 16 weeks of age, tends to disappear entirely after that.

Rate of Growth

The mean rate of growth of the shanks of the different subgroups was computed by multiplying the differences between the natural logarithms of successive shank-length measurements by 100 (to give the percentage) and dividing them by 4 (number of weeks between measurements). The values thus obtained represent then the rate of increase in the means rather than the mean rate of increase. They are presented in Table 4.

TABLE 4

Average Weekly Percentage Growth Rate Computed from Mean Shank Lengths

Line	Ration	Period					
		4-8 wks.	8-12 wks.	12-16 wks.	16-20 wks.	20-24 wks.	24-28 wks.
Size	*A*	10.28	6.60	5.03	2.11	0.36	0.09
	B	9.59	7.13	4.72	1.46	0.22	0.05
Production	*A*	9.97	6.29	4.19	1.42	0.26	0.08
	B	9.64	6.30	4.08	1.00	0.19	0.05

It is clear from this table that: (*a*) the rate of growth in all subgroups was, as expected, ever-decreasing; (*b*) the Size line had a higher rate of growth than the Production line at every age with the exception of the first and last period on Ration *B;* (*c*) the effect of the ration differences on growth antedates the earliest measurement made. Only the latter point requires a specific comment. With the exception of a reversal between 8 and 12 weeks of age the rate of growth of the shank is uniformly higher on Ration *A*. This is obviously a case of compensatory growth. The growth-promoting advantages of Ration *B* have exercised their influence prior to four weeks of age, after which the compensatory effect leads to an increased growth rate of the birds on Ration *A*. Similar situations due to seasonal and hereditary causes, rather than to dietary ones as in this case, have been previously described by Lerner and Asmundson (1938).

From the standpoint of genetic differentiation in size it is thus clear that Path *b* as listed in the introduction (differential rate of growth) contributes significantly to the differences between the two lines.

Duration of Growth

Jaap (1941) has shown that the shanks of chickens reach their maximum size between 18 and 22 weeks of age. There was, however, some indication in his material that the Brahma, which is a heavy breed, takes somewhat longer to achieve its mature shank length than a smaller breed such as the Leghorn. A similar inference may be drawn from Lerner's (1941) data on Leghorns and Bantams, namely that the larger birds have a longer duration of growth. This refers, of course, to the *linear growth of the shank* only, and is in no way contradictory to the findings of Waters (1931) with regard to the uniformity of duration of *growth in body weight* in birds of different mature size.

Unfortunately, the precision of measurements possible on living birds is not great enough to permit an exact evaluation of the effect of possible differences in duration of growth between the Size and the Production lines. It is clear, however, from Table 4 that the negative acceleration in the rate of growth of the Size line is so great that such an effect cannot be particularly significant. From Table 5, which lists the percentages of birds which failed to show gains at the successive measuring times, it would also seem that little or no difference

TABLE 5

PERCENTAGE OF BIRDS COMPLETING GROWTH OF SHANK BY THE SPECIFIED AGE

Line	Ration	Age in weeks 16	20	24	28
Size	*A*	0	0	30.0	70.0
	B	0	3.8	50.0	88.5
Production	*A*	0	11.6	46.4	79.7
	B	0	10.8	54.1	77.0

between lines in the duration of growth exist. Finally, the fact that practically all of the difference in length of shank (as may be seen from Table 1) is reached by 20 weeks of age when only a very small percentage of the birds have completed their growth in shank length, would definitely indicate that Path *c* (differences in duration of growth) is of little or no consequence in establishing the observed difference.

ORIGINAL LENGTH OF SHANK

It now remains to examine Path *a* (original size differences) with respect to its possible effect on size differentiation. From the standpoint of the dietary differences it clearly has no effect. The segregation into lots was made at the time of hatching and the offspring of each hen were distributed as equally as possible between the two lots fed different diets. It is obvious that no difference in the length of the shank of day-old chicks on Rations *A* and *B*, nor in size of the original zygotes could be expected to exist outside of sampling fluctuations.

With respect to the differences between lines this is not as clear. The first possible differential may lie in a difference in egg size between the two lines which would affect chick size. However, as it happens, the difference is in the opposite direction. The spring egg weight of birds on the two lines does not differ in their pullet year (unpublished data). The Size line in the particular year of the experiment originated entirely from pullets; the Production line entirely from hens. Hence the effect of egg size would favor the smaller rather than the larger line.

That this is a likely situation and that, consequently, initial size differences do not contribute to the size differentiation between lines, may be seen from the following computations. It is possible to calcu-

late the necessary difference in rate of growth between the Size (R_s) and the Production (R_p) lines from 0-4 weeks of age to account for the observed difference between the mean shank lengths of the two lines at the latter age by solution of two simultaneous equations. For Ration *A* these equations are (with *R* and the appropriate subscripts as defined above, and $L_s = L_p$, standing for the assumedly equal shank lengths at hatching time of the respective lines):

$$log_n 3.97 - log_n L_s = 100\, R_s/4$$
$$log_n 3.92 - log_n L_p = 100\, R_p/4.$$

For ration *B* the equations are:

$$log_n 4.17 - log_n L_s = 100\, R_s/4$$
$$log_n 4.06 - log_n L_p = 100\, R_p/4.$$

The respective solutions for the two rations are:

$$A). \quad R_s - R_p = .0006$$
$$B). \quad R_s - R_p = .001.$$

Thus it is clear that even taking the conservative assumption that the shank lengths at hatching time were the same in the two lines, a very minute differential in rate of growth between them would account for the observed difference at four weeks of age. It is most likely, however, in view of these figures and the values in Table 4, that the initial length difference was in favor of the Production line.

Conclusions

It appears then that the differentiation of shank length in the genetically distinct lines is due to cumulative differences in rate of growth rather than to initial size differences or those in duration of growth. Similar differentiation in the early growth periods due to dietary differences is accounted for by growth rate divergences from hatching time to four weeks of age.

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THE GROWTH CONCEPT OF NERVOUS INTEGRATION: V. THE THEORETIC FORMULATIONS AND THE BASIC EQUATIONS FOR THE RELATIONS OF HEAT PRODUCTION, BODY MASS, BODY LENGTH, BODY AREA, BRAIN WEIGHT, CORD WEIGHT, AND ELECTROENCEPHALOGRAPHIC FREQUENCY. BASIS FOR AN ELECTROENCEPHALOGRAPHIC METHOD OF DETERMINING BASAL METABOLISM*

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In the introductory paper of this series (1), the growth concept of nervous integration was outlined and its basic mathematic formulae presented. It was stated that when growth was treated as an electrochemical process in which the brain and cord weight (specific to each species) were considered as representations of the species forces of growth, it was possible for the first time to demonstrate the dynamic relationship between somatic growth change in mass, heat production, length, cross-sectional area, and nervous growth, including the growth increase of the alpha frequency of the electroencephalograph.

This was made possible by utilizing Loeb's rather than Sherrington's concept of nervous cellular function and integration. Loeb (2) conceived of the nerve-cell as acting like a "relaxation-oscillator." Hoagland (3) demonstrated the validity of this concept electroencephalographically in 1936, 36 years after Loeb first announced it.

Briefly, Loeb's concept led one to conceive of the whole organism, from the moment of fertilization, to be under the impact of a growth *electromotive* force, and to think of the life-process as a manifestation of the function of *irritability* which in the higher species received more and more tangible and complex mechanization in the organization of the nervous tissue. From this, it followed that all the transactions of the organism could be viewed, in accordance with thermodynamic law, as either potential or kinetic energy transactions, their sum total amounting to the thermodynamic work of growth. Wetzel

*Machine computations by Dr. Benjamin Balinsky, College of the City of New York.

(4) had already confirmed Tangl's conception of a thermodynamic work of growth. Since these transactions could be none other than variations of irritability and growth electromotive force operating in and through irritability, then to the nervous system must accrue the forces of growth and to its lot must fall the task of integrating the potential and kinetic energy transactions of growth at any given time.

Thus:

$$\textit{Thermodynamic Work} = \textit{Potential Energy} + \textit{Kinetic Energy} \quad (1)$$

Or:

$$\textit{Thermodynamic Work of Growth} = \textit{PE of Growth} + \textit{KE of Growth} \quad (2)$$

Put in another way, the organism, in growth and function, could be considered as an amalgamation of *tension* processes (determined by potential energy transactions) and *relaxation* processes (determined by kinetic energy transactions). This new way of phrasing the problem was already encouraging since medicine had only recently begun to describe under a definite heading, the *tension-syndromes*, from vascular hypertension to ulcer, migrain, etc., not even excluding carcinoma so intricately bound up with the estrogenic tensors, which are sterol derivatives.

Next, since we could consider the entire organism as well as the neuraxis a conductor, Joule's Law was applied, or:

$$\textit{Heat} = \textit{Work} = i^2 Rt \quad (3)$$

where i is the current intensity, R the resistance, and t the time of passage of current.

Since the heat of the organism at any time could be given by:

$$\textit{Heat} = \textit{Mass} \times \frac{\textit{heat}}{\textit{mass}} \quad (4)$$

then,

$$\textit{Heat} = \textit{Mass} \times \frac{\textit{heat}}{\textit{mass}} = i^2 RT. \quad (5)$$

Since $i = \frac{E}{R}$

$$\textit{Heat} = \textit{Mass} \times \frac{\textit{heat}}{\textit{mass}} = i^2 Rt = \left(\frac{E}{R}\right)^2 . Rt = \frac{E^2}{R} t \quad (6)$$

Therefore,

$$Mass \times \frac{heat}{mass} \times R = E^2t \tag{7}$$

Since Faraday's Law for electrolytic chemical systems permits the expression of mass as:

$$Mass = z \cdot i \cdot t \tag{8}$$

where m is mass, z is electrochemical equivalent, i is current intensity, and t is time, the formulation of Equation (7) now represents a system acting both electrochemically and as a conductor which fulfills two important known functions of animate organisms.

At this point we are able to make the distinction between growth heat and maintenance heat, thanks to the brilliant work of Wetzel (4), whose whole concept however was inapplicable because his was a system dealing solely with the dimension of log_e *mass* or q, the quantity of growth, and furthermore took no account of the nervous system as a *determining* factor. [This, we believe, is responsible for the slight but significant deviation of Wetzel's theoretic heat curve, between the ages of two to five, as compared with the empiric data of Scammon (5), Benedict (6), and others.]

Nevertheless, students of growth are indebted to Wetzel for the neat dissection of heat production into these two component parts: the heat of growth, the dynamic component; and the heat of maintenance, the non-dynamic component needed to maintain life. Or, in other words, the "circuit" in the living organism, as a conductor and as an electrochemical unit, is not made of durable copper but of organic conductive and operative material which has constantly to be refurbished in the "wear and tear" of sheer existence.

Thus, Equation (7) may be considered as taking two forms, accepting Wetzel's value for the heat of maintenance per unit mass as 25.3425 calories per kilo.

$$Mass \times \frac{Maintenance\ Heat}{mass} \times R_{(m)} = E^2_{(m)}t \tag{9}$$

and

$$Mass \times \frac{Growth\ Heat}{mass} \times R_{(g)} = E^2_{(g)}t \tag{10}$$

the subscripts (m) and (g) referring now to maintenance and growth respectively and x referring to the variable nature of $E_{(g)}$. Equation (10) was described in the introductory paper as the "basic equation," thus:

Growth Heat × *Growth Resistance* = (*Growth Electromotive Force*)t

Since $R = \rho \frac{L}{A}$ where ρ is the specific resistivity of the conductor, L its length, and A its cross-sectional area, we now have accessible all the variables and need only the formulation of nervous mass as growth electromotive force.

There are two main dispositions of nervous mass anatomically: cord mass and brain mass. Functionally, however, we conceive of the growth electromotive force as operating in two main forms through nervous mass: the first, the so-called "decerebrate" organization encountered in the living organism under such conditions as sleep and coma in which the practical disappearance of the E.E.G. wave indicated that the cord weight alone could represent (without too great an error) one organized disposition of force; the second, that of the waking state in which both brain and cord operate together, not simply additively, but as "transformers," i.e., exponential to each other.

Thus, growth electromotive force was represented as:

$$(E)^{x_{1(g)(1)}}\, t \propto \textit{cord weight} \tag{11}$$

and:

$$(E)^{x_{(g)(2)}}\, t \propto \textit{brain weight}^{\log_e \textit{cord weight}} \tag{12}$$

where the exponent takes the logarithmic form.

Equation (10) itself hence takes two forms corresponding to the two dispositions of growth force represented by Equations (11) and (12). Or:

$$(\textit{Mass})^{x}\left(\frac{\textit{Growth Heat}}{\textit{mass}} \times R_{(g)}\right)^{y} = \textit{brain weight}^{\log_e \textit{cord weight}} \tag{13}$$

and:

$$(\textit{Mass})^{x_1}\left(\frac{\textit{Growth Heat}}{\textit{mass}} \times R_{(g)}\right)^{y_1} = \textit{cord weight} \tag{14}$$

where x, y, x_1, and y_1 are exponents corresponding to the dimensions of $E^{x}{}_{(g)}t$ and $E^{x_1}{}_{(g)}t$ respectively.

There now remained only the necessity to distinguish between maintenance resistance, $R_{(m)}$, and growth resistance, $R_{(g)}$.

When growth has "ceased," i.e., when it has reached adult equilibrium, there are no further significant changes in length (until senility, when length may decrease slightly). A maintenance level will then have been achieved and resistance at this state will be given simply by $\rho \frac{L}{A}$ which, for all practical purposes, may be considered constant.

While growth is going on, there will be a change in the three directions, length, transverse diameter, and anterior-posterior diameter; the changes in these directions will accompany any change in mass per unit time.

Thus for any increment in mass, say $\Delta(m)$, there will be a corresponding $\Delta(L)$, $\Delta(A_1)$, and $\Delta(A_2)$ where the subscripts refer to the two diameters of cross-sectional area. These data may be obtained from Meredith's (7) excellent study.

For any increment of mass per unit time, the change in the dimensions of the resistance of the increment becomes:

$$\frac{\Delta(L)}{\Delta(A_1) \times \Delta(A_2)}$$

At the same time, while the change in the relationships of the increment is taking place, changes in the total length and the total area are also occurring so that during growth $\rho \frac{L}{A}$ is not constant. Furthermore, a significant alteration in resistance will take place only so long as the ratio

$$\frac{\frac{\Delta L}{\Delta(A_1) \times \Delta(A_2)}}{\rho \frac{L}{A}}$$

is not constant, since at maintenance the fraction

$$\frac{\Delta L}{\Delta(A_1) \times \Delta(A_2)}$$

will be unity and $\rho \frac{L}{A}$ will be constant.

Hence growth resistance can be adequately represented by:

$$\frac{\frac{\Delta(L)}{\Delta(A_1) \times \Delta(A_2)}}{\rho \frac{L}{A}} = R_{(g)} \tag{15}$$

Equations (13) and (14), expressed in natural logarithms, now become, letting H_g represent growth heat per unit mass:

$$x \log_e mass + y\,[\log_e H_{(g)} + \log_e R_{(g)}] + C =$$

$$(\log_e Br.\,Wt.)(\log_e Cd.\,Wt.) \tag{16}$$

$$x_1 \log_e mass + y_1\,[\log_e H_{(g)} + \log_e R_{(g)}] + C_1 = \log_e Cd.\,Wt. \tag{17}$$

Since alpha frequency of the electroencephalogram follows the growth of the brain, it may be expressed as

$$\alpha\ Frequency = A\ (Brain\ Weight) + B \tag{18}$$

Or

$$\alpha \frac{Frequency - B}{A} = Brain\ Weight \tag{19}$$

Then, by dividing Equation (17) into Equation (16), we obtain:

$$\frac{x \log_e mass + y\,[\log_e H_{(g)} + \log_e R_{(g)}] + C}{x_1 \log_e mass + y_1\,[\log_e H_{(g)} + \log_e R_{(g)}] + C_1} =$$

$$\log_e Br.\,Wt. = \log_e\left(\frac{\alpha F - B}{A}\right) \tag{20}$$

Equation (20) may now be tested from the empiric data. The exponents are derived by the method of expansion of determinants.

For the male values, using an average of various weight curves in the literature as our basic data, the constants are:

$x =$	5.59175473	$A =$	0.00836557
$x_1 =$	0.68545936	$B =$	−1.39910521
$y =$	0.43740094		
$y_1 =$	0.04971345		
$C =$	−0.39333199		
$C_1 =$	0.33581311		

TABLE 1

Age years	Mass kilos	Heat/mass* Cals./Kg./yr.	$log_e R_{(g)}$	Brain wt. (grams)	Cord wt. (grams)**	Alpha freq./sec./yr.	Total heat cals./yr.
Birth	3.25	(43.25) (50.71)	1.4800	(386.81) (400.68)	(3.90) (3.97)	1.95	(140.56) (164.80)
½	7.57	55.66	2.6370	816.38	7.57	5.43	421.15
1	10.06	54.44	2.9270	937.91	9.31	6.45	547.67
2	12.70	51.17	3.5943	1045.52	11.22	7.35	649.86
3	15.04	48.28	5.2483	1164.30	13.61	8.34	726.13
4	16.71	46.24	4.9094	1182.71	14.31	8.49	772.67
6	20.45	42.60	3.7978	1199.28	15.40	8.63	871.17
—	——	46.94	——	1207.59	15.57	8.70	959.92
8	26.34	39.05	3.6656	1265.32	18.00	9.19	1027.58
—	——	41.64	——	1271.52	18.16	9.24	1096.80
10	32.53	35.99	3.4032	1310.53	20.31	9.56	1170.75
—	——	37.83	——	1316.18	20.47	9.61	1230.60
12	38.68	34.99	3.1106	1346.22	22.39	9.86	1353.41
14	48.62	31.40	2.9963	1389.86	25.45	10.23	1526.67
16	56.00	29.21	3.1156	1416.76	27.59	10.45	1635.76
18	63.00	27.78	1.4098	1377.12	26.85	10.12	1750.14
	63.5	27.6378	1.2577	1372.33	26.71	10.08	1755
	64.0	27.5000	1.2656	1372.60	26.78	10.08	1760
	64.5	27.4109	1.2734	1373.56	26.87	10.09	1768
	65.0	27.2615	1.2810	1373.42	26.93	10.09	1772
	65.5	27.1756	1.2887	1374.25	27.02	10.09	1780
	66.0	27.0606	1.2963	1374.38	27.08	10.09	1786
	66.5	26.9474	1.3038	1373.56	27.14	10.09	1792
	67.0	26.8657	1.3113	1374.93	27.22	10.10	1800
	67.5	26.7556	1.3188	1374.79	27.27	10.10	1806
	68.0	26.6176	1.3262	1373.56	27.28	10.09	1810
	68.5	26.5401	1.3335	1373.42	27.34	10.09	1818
	69.0	26.4058	1.3408	1372.05	27.32	10.08	1822
	69.5	26.3165	1.3480	1370.96	27.35	10.07	1829
	70.0	26.1857	1.3551	1368.22	27.28	10.04	1833
	70.5	26.0993	1.3623	1367.53	27.29	10.04	1840
	71.0	25.9859	1.3693	1364.25	27.22	10.01	1845
	71.5	25.8741	1.3763	1360.14	27.10	9.98	1850
	72.0	25.7639	1.3833	1354.73	26.93	9.93	1855
	72.5	25.6552	1.3903	1347.16	26.66	9.87	1860
	73.0	25.5479	1.3971	1335.47	26.24	9.77	1865

*To obtain growth heat per unit mass, subtract 25.3425 (Wetzel's constant) from heat per unit mass.
**Cord weights measured without nerve-roots attached.

The heat values used in this paper are, more correctly, lower than those utilized in the introductory paper. The present values are from Benedict's (6) data for boys from 1 to 13 years of age, and Bierring's data for weight and heat from 20 kilograms to 73 kilograms. The tables show the results, with two solutions for those values where Benedict's and Bierring's data overlap. The values from 63.5 kg. to 73 kg. represent various adult stages at which growth may reach equilibrium.

The formulation seems to hold so accurately with variations obviously dependent on the exact value of weight: heat relationships, that it now should become possible to measure basal metabolism from electroencephalographic frequency, using Equation (20).

Finally, from the theoretic point of view, this whole concept must be further substantiated by showing that the heat of growth can be derived, in an electrochemical formulation, from the changes in the rate of cellular synthesis and in resistance, as Wetzel (4) has already demonstrated. This is being done and will be reported in a subsequent communication.

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FISSION RATE IN DERO AS INFLUENCED BY B-COMPLEX VITAMINS, PARASITISM, AND SEXUAL CYCLE*

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The following preliminary study is the outcome of prolonged laboratory cultivation of Dero limosa and an unidentified Dero species. These aquatic oligochaetes have been kept in culture for intermittent periods over three years, during which time the cytology and host-parasite relationship of the coccidian, Adelina deronis, was the principal aim of investigation (1). The potential usefulness of naids for growth-inquiry was evident. In preparation for future analysis of the rôle of certain amino acids, vitamins, and carcinogens in regenerative growth, several genetically pure strains of Dero have been developed since October, 1943. Knowledge of normal growth rates and the attainment of relatively standardized material were the object of the present work.

A. MATERIAL AND METHODS

Parasite-free as well as infected individuals of Dero limosa and of a naturally immune large Dero species were collected from a pond in the University of Pennsylvania botanical garden. Cultures were derived from single specimens grown at room temperature on boiled lettuce (2) in covered stender dishes containing 30 cc. of filtered spring water. Three varieties of lettuce were tested. Romaine lettuce was found most satisfactory. The medium was renewed every three or four days.

The presence of the coccidian, Adelina deronis, infesting about 20 per cent of the worms gathered during the autumn months of 1942 and 1943, could be reliably determined. The very transparent hosts were examined in vivo at a magnification of 400 diameters. When found negative, they were quarantined for 18 days—equivalent to the duration of the parasite's complete life cycle—and re-checked at

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the end of this period, whereupon a culture could be labeled as parasite-free with certainty.

To insure against accidental contamination with Adelina oöcysts, sterile pipettes, glass-ware, and needles were employed in the routine procedures.

When properly fed and not too severely infected, the worms divide by transverse fission. This vegetative budding results in a genetically uniform stock, if the medium is inoculated with a single animal. Strains thus derived were allowed to multiply for several weeks and were then divided into equal portions to serve as tests and controls in the B-complex study.

The cultures were sufficiently small to facilitate accurate counting (by means of a stereoscopic microscope) of all the individuals and fission zones present.

The eight B-complex vitamins used were made available through the kindness of Dr. M. A. Bennett. The relative proportions were the same as those in rat nutritional studies by Bennett and Toennies (3), but the concentration differed as follows: To 30 cc. of filtered spring water were added thiamine hydrochloride, riboflavin, pyridoxine hydrochloride 4γ each, calcium pantothenate 20 γ, nicotinic acid, p-aminobenzoic acid, and inositol 50 γ each, biotin methyl ester 0.025 γ, ethanol 0.0005 cc. (A negligible amount of sucrose was present in the mixture. Choline was intentionally omitted).

For the determination of fission-zone-position and segment counts, the worms were anaesthetized in 0.2 per cent chloretone from which they readily recovered.

B. Results

1. *The Influence of B-Complex Vitamins*

Figure 1 shows growth progression for a test and a control series of Dero limosa and, likewise, for the considerably larger unidentified Dero species. The latter differs from the former in size, length of setae, number of segments, and other minor morphological details in addition to such physiological characteristics as growth-rate, natural immunity to Adelina infections, and sexual cycle.

All strains shown in Figure 1 were parasite-free. The log. of the number of individuals is plotted against time. Since the two sister zoöids in a worm with a fission-zone separate soon after the zone has become visible, the tandems were counted as two individuals.

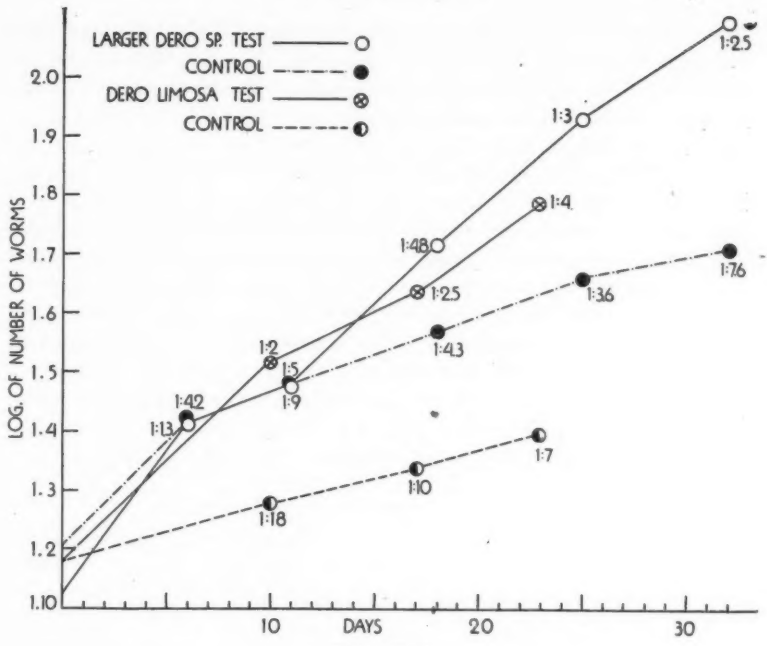

FIGURE 1

EFFECT OF B-COMPLEX VITAMINS ON THE GROWTH OF TWO SPECIES OF DERO
Numbers indicate ratio of dividing to non-dividing animals.

The ratio of dividing to non-dividing worms at various intervals is given along each curve.

The vitamin-fed lot of the large Dero sp. exhibited no significant deviation from the control for the first 11 days. The test was started with 14 animals, the control with 16. On the 11th day both cultures (which were genetically identical) contained 30 worms. From then onward the two curves diverged until on the 32nd day the test was 144 per cent ahead of the control. On the last day of this experiment the division frequency in the vitamin-series was three times that in the control (1:2.5, as against 1:7.6).*

The apparent latent period of response to the vitamin medium is in part the result of the short interval chosen for the determinations

*Sixty-three days later the vitamin-fed cultures of the large Dero sp. contained 1,190 individuals as against 236 in the controls.

(an interval of about one-half the generation time). No such lag was recorded for the Dero limosa test which, on the 10th day of the experiment, was already 68 per cent ahead of the control. On the 23rd day this difference was widened to 144 per cent, the same divergence which had been attained by the large Dero series in 32 days.

Growth in Dero limosa, therefore, seems to be more closely dependent on B-complex vitamins than in the other species tested.

In Figure 1, increase is recorded in terms of individuals. Does this at the same time imply an increase in mass? While there were a few dwarfed animals in the vitamin-starved cultures, the specific sizes were, on the whole, quite comparable in tests and controls.

The actual mass relationships are best shown by a comparison of segment numbers. For instance: 20 test-worms (large Dero sp.) chosen at random totaled 1,210 segments, compared with 1,223 segments in 20 control worms. This surprisingly close agreement may be the consequence of genetic identity. The segment averages in Table 1 are revealing.

TABLE 1

	Large Dero sp.		Dero limosa	
	Test	Control	Test	Control
Average segment number in worms *without* fission zones	60.5	61.2	43.9	39.6
Average segment number in worms *with* fission zones	82.8	80.0	57.2	67.0

Only very few animals with fission zone were present in the Dero limosa control. The average segment number for this group (67.0) is probably not characteristic.

In general, there are *no* significant differences between tests and controls in the two species. Since segment size is specific and segment number agrees so closely, the number of individuals serves as a fairly reliable index of the mass aspects of this growth problem.

A comparison of segment numbers of anterior and posterior zoöids separated by a fission zone showed close agreement (Table 2).

TABLE 2

Average number of segments	Large Dero sp.		Dero limosa	
	Test	Control	Test	Control
In anterior zoöid (*n*)	40.4	42.3	29.5	32.0
In posterior zoöid	41.4	41.0	27.3	35.0

This tabulation indicates that the fission zone in both species develops approximately in the middle of the body, as far as segment number is concerned. The number of segments of the anterior zoöid (*n*) is slightly higher in the controls. This coincides with Stolte's view, as quoted by Stephenson (4), that food is the principal factor governing the length of the zoöids, *n* being inversely related to the state of nutrition.

All the averages given above are close to the modes, since the range in segment number is relatively narrow and the frequency distribution within this range follows a steep normal curve.

Calculation of percentage increase of segment-number (rather than individuals) indicates that the vitamin effects in the two species are of the same order of magnitude—a point of considerable interest for further analysis.

2. *The Influence of Parasitism*

As evident from Figure 2, Adelina infections have only a slight de-

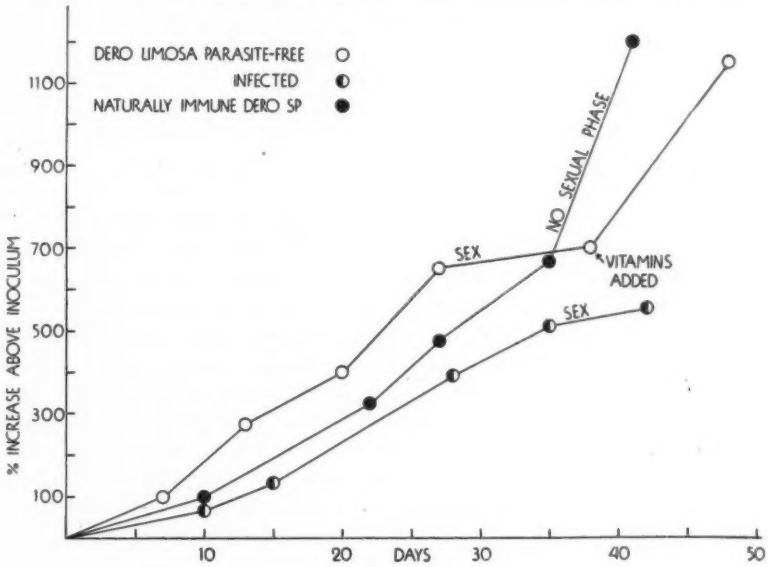

FIGURE 2

EFFECTS OF PARASITISM AND SEXUAL PHASE IN DERO GROWTH

pressing effect on the growth rate of the host. On the 39th day of the experiment the uninfected cultures showed a 700 per cent increase over the original inoculum, while there was 525 per cent increase in the parasitized lot.

The sporozoön Adelina is extremely efficient in its relationship to Dero limosa. Infections are seldom lethal. Their transmission is not restricted to the ingestion of oöcysts. The posterior zoöid usually inherits the parasite via the fission zone from its anterior sister. Forty-two days after isolation of eight infected specimens in eight separate dishes, 57 worms showed 51 of these 57 animals to be infected. As there had been no deaths in the eight cultures during the period mentioned, the remarkable spread of the disease was by way of adjoining somites which were later separated by the fission zone.

The Adelina factor has a rather variable effect on growth, depending on the initial dosage of parasites which have invaded the host. Eight parasite-free isolation lines, each derived from a *single* animal, showed the following increase after four weeks: 5, 6, 6, 7, 7, 7, 8, 8 (average, 6.75; range 5-8). This served as control for the eight infected lines—also derived from single worms—which after four weeks contained 1, 2, 2, 4, 5, 6, 7, 8 individuals (average 4.37; range 1-8). One of the infected worms had failed to divide, two had divided only once in four weeks, while the fission rate of the rest was comparable to the control. Close correlation was found between severity of infection and fission frequency.

Cultures of the large Dero sp., though experimentally fed enormous quantities of Adelina oöcysts, over a period of several months, could not be infected. This form must, therefore, be considered as naturally immune. Four other naid genera proved likewise uninfectable.

The successful adaptation of the parasite to Dero limosa revealed by a rather negligible depression of host-fission-rate and a high degree of host-specificity suggests a phylogenetically very ancient association.

Also the great variation in severity of infection, as expressed through the host's fission frequency, furnishes indirect evidence for the self-limited nature of the sporozoan life-cycle (1). Unlimited schizogony would seriously hamper prolific multiplication of the host. The variability may be explained on the basis of varying intakes of infectious material.

3. *The Influence of the Sexual Cycle*

No sexually mature animals appeared in the cultures of the large Dero sp. Taxonomic identification was therefore not feasible. The growth-curve of this species (Figure 2) does not show the break exhibited by both the infected and uninfected Dero limosa strains. This levelling-off was brought about by the appearance of sexually mature individuals in which fission zones were very rare. Most of the mature forms died after a few days.

Two weeks after the first appearance of sexuality, the surviving immature forms resumed vegetative fission at a reduced rate, which was boosted to resume its pre-sexual slope by the addition of B-complex vitamins in the above-described concentration. No vitamins were added to the infected strains which began to die off after 42 days of culture. The decline in this case was due to the combined inroads of sexuality, vitamin-starvation, and parasitism, against which even the extraordinary regenerative capacities of Dero limosa were of little avail.

C. Discussion

Investigation of vitamin requirements in animals has been limited largely to the higher vertebrates, a few insects, and several Protozoa. Most of the other invertebrate phyla are not mentioned in the literature. From the preliminary findings reported above it appears that the naid oligochaetes could be useful in this connection. Growth studies on these forms employing a synthetic medium are not beyond possibility. But here, as in most of the metazoa, the complications introduced by the intestinal flora may raise great difficulties.

The medium used in the above study contained only boiled lettuce in addition to the vitamins. Lettuce is very low in protein, lower than any other vegetable in carbohydrates (other than fiber), low in fat, intermediate in calcium, phosphorus, iron and copper content. Compared with other vegetables, it is approximately average as a thiamin, riboflavin and niacin source (5). With regard to the other B-complex vitamins, lettuce is comparatively low in content of pantothenic acid, above average in pyridoxin and biotin, and very high in inositol (6). These comparative values are based on dry weight. Since lettuce has a dry weight of only 5.2 per cent, relatively large quantities must be eaten to satisfy certain nutritional requirements.

The vitamin fed tests attained their greater fission frequency through consuming *more* lettuce. The vitamins as such do not account for the mass-difference.

No correlation was found between vitamin starvation and the sexual cycle in Dero limosa. Sexually mature animals appeared at about the same time and in about the same proportion to total population in tests and controls. Parasitism also did not interfere with gonad development.

Some of the other physiological and ecological aspects of fission in naids are exhaustively and ably discussed by Stephenson (4) who also lists a complete bibliography.

D. Summary

1. Cultures of Dero limosa and of an unidentified Dero sp. exhibit signs of decline when grown on scalded lettuce alone.

2. A synthetic medium containing eight vitamins of the B-complex in known concentration greatly accelerates the fission rate in isolation-line strains of two Dero species.

3. Adelina deronis infections, unless very heavy, do not seriously depress fission rates of the host.

4. The sexual phase is accompanied by a drop in fission rate.

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GROWTH OF ALBINO RATS ON A CHOLINE-FREE DIET IN WHICH HOMOCYSTINE IS THE ONLY SULFUR-CONTAINING AMINO ACID*

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A. INTRODUCTION

The work of du Vigneaud and associates, according to which the effectiveness of homocystine in replacing methionine in the diet of growing rats depends upon the presence of choline or related methyl donors (2, 3) opened many new possibilities for metabolic investigation. Results of du Vigneaud *et al.* (4) agree with the hypothesis that demethylation of methionine to homocystine is an intermediary step in the conversion of methionine to cystine. On the other hand, the finding of Floyd and Medes (5) that in tissue slice experiments methionine is more effective than homocystine as a precursor of cystine supports the view (6) that labile methyl groups may be required in the process. The present investigation began as an attempt to obtain evidence on this problem by means of rat growth studies. However, the unexpected outcome of the first experiments made it necessary to defer the original question and led instead to an investigation of the phenomenon stated in the title.

B. COMPOUNDS

The composition of the amino acid mixture used (Table 1) is based on the data of du Vigneaud *et al.* (2) and of Rose and Wood (7). All amino acids except hydroxyproline (University of Illinois) and arginine monohydrochloride (Amino Acid Manufactures), were products of Merck and Company. Application of the method of Lavine (8) to this mixture gave a methionine value corresponding to —0.01 per cent of the total diet, or, after correction for the effect of the known tryptophane content, +0.03 per cent.

*Aided by a grant from Mrs. L. Elizabeth Nax. For preliminary accounts of parts of this work, *cf.* (1).

TABLE 1
COMPOSITION OF AMINO ACID MIXTURE

	Final per cent in diet
Glycine	0.1
dl-Alanine	0.4
dl-Valine	2.0
dl-Leucine	2.4
dl-Isoleucine	1.6
l(—)-Proline	0.2
l(—)-Hydroxyproline	0.1
dl-Phenylalanine	1.2
l(+)-Glutamic Acid	2.0
dl-Aspartic Acid	0.4
dl-Serine	0.2
l(—)-Tyrosine	0.6
l(—)-Histidine, $HCL \cdot H_2O$	0.7
l(+)-Arginine·HCl	0.5
dl-Lysine·HCl	3.0
l(—)-Tryptophane	0.4
dl-Threonine	1.4
$NaHCO_3$	1.9
Total	19.1

For the determination of choline, a mixture of 8.0 grams of the basal diet and 1.3 grams of the washed butter (corresponding approximately to the amount consumed in two days, *cf.* Diet) were extracted with alcohol-ether (9), the evaporation residue of the extract was refluxed for three hours with 10 cc. 1 *N* H_2SO_4 (10) in a stream of nitrogen, the aqueous layer with several washings was neutralized with $Ba(OH)_2$, and, after removal of $BaSO_4$ and slight acidification with acetic acid, was evaporated to 10 cc. Precipitation with reineckate (11) of aliquots with and without added known amounts of choline indicated a content of less than 0.5 mg. choline for the portion (9.3 grams) analyzed.

The cystine and methionine were Merck's Reagent grade.

Homocystine was obtained from the S. M. A. Corporation. Each lot was assayed by colorimetric disulfide determination (12), using pure l-cystine as the standard. The values found averaged 97.5 per cent of the theoretical.

Modified ryzamin B was prepared according to du Vigneaud and associates (2). If the reineckate precipitate obtained in this procedure is assumed to be choline reineckate the choline content of ryzamin B is 1.3-1.35 per cent (4 determinations). However, the periodide

method (10) gave values of only 0.4-0.5 per cent for free choline and 0.8-0.85 per cent for total choline (after four hours' hydrolysis with 1 *N* HCl). The combination of modified ryzamin B, thiamin, riboflavin and niacin (2), as well as, in a few cases, the combination of thiamin, riboflavin, niacin, and pyridoxin of Chandler and du Vigneaud (13) were administered in the same manner as, and in place of, the combination of eight B-vitamins (14).

C. Diet

The methionine- and choline-free basal diet had the following percentage composition: synthetic amino acid mixture (Table 1) 19, dextrin 30, sucrose 15, salt mixture 4 [Osborn and Mendel (15)], agar 2, and Mazola corn oil, 30. The corn oil as well as the butter were thoroughly washed with acid and water to free them from traces of choline. Water soluble vitamins (*cf.* Table 7) were given as previously described (14), their administration beginning with the basal diet. Except for the incorporation of vitamin K (16) the vitamin content of the basal diet[1] was substantially as in previous work (14). The basal diet was fed *ad libitum* unless otherwise stated. Amino acid supplements were mixed with butter as previously (14) described, and fed in this form, the daily butter ration being approximately 0.6 grams per rat. For the administration of choline, emulsions of the chloride in unwashed (moist) butter were prepared with the aid of heat and shaking, in such concentrations that the daily portion corresponded to 0.1 cc. of the emulsion. This was added to the amino-acid butter supplements.

D. Animals

Female albino rats, Wistar strain, five weeks old, were used as experimental animals, since it has been reported (17) that females of that age are more resistant to choline deficiency disturbances than males or younger females. No more than eight rats were raised in a litter. The pre-experimental (general colony) diet consisted of: liver, cheese, fish, eggs, milk containing Squibb's Navitol and Zygon,

[1]Per 1,000 gms. of basal diet: 28.1 mgs. of carotene (90% β—, 10% α—, SMACO), 11.3 mgs. of 2-methyl-1,4-naphthoquinone (SMACO), 619 mg. of viosterol in oil (Squibbs, 10,000 USP units of vitamin D per gram), and 16.3 mgs. of α-tocopherol (SMACO).

yeast, bone meal, oats, checkers, carrots, lettuce, cabbage, tomatoes, and oranges. Each cage had a salt ring. The general management of the animals during the experiments was as previously described (14) except that weighings were made daily. The animals were kept in separate cages during all significant periods. They were handled entirely by one person throughout the investigation.

During this work, covering more than 3,000 animal-days, only one rat (third series, *B*17), was observed on two days (the first two after supplementation with choline began) to eat its feces. This fact, however, does not rule out nocturnal coprophagy.

First experimental series. This series consisted of 14 rats, selected from 27 animals from four litters born within two days: 3, 6; 8, 11; 15, 16, 18; 20-23, 25-27. The criterion of group uniformity was the growth constant K of Zucker *et al.* (18) which was calculated for the individual rats from their weight changes during the first twelve days after weaning.

Second experimental series. This series consisted of 18 rats, selected from 24 animals from four litters born within 2 days: 1-7; 8-13; 18-21; 22. They were divided into three groups of six rats each to obtain group averages equal with respect to weight and Zucker's growth constant. In the supplementary experiment of four animals, 41 to 43 were sisters, 48 was born one day later.

Third experimental series. This series consisted of 16 rats selected from 21 originating from four litters born on the same day: 1, 4-6; 7, 8, 10, 11; 12, 13, 15, 17; 18-21. They were arranged in four groups (each including one animal from each litter) comparable in their averages with respect to weights and growth rates, as shown in Figure 5.

E. Growth Experiments

1. *First Series*

The plan of the first exploratory series was to feed to a group of rats a low amount of methionine (0.067 mM) which it was expected would cover the requirement of the animal for methionine *per se*. Additional supplements of methionine, homocystine or cystine were administered to individual animals in order to obtain a comparison of their relative effectiveness in supplementing the minimal methionine supply. By this method it was hoped to demonstrate which of

TABLE 2

SUMMARY OF RESULTS OF FIRST EXPERIMENTAL SERIES

Animal number	Desig-nation	Daily supplements				Weight of animals			
		Methionine mM	Cystine mM	Homocystine mM	Choline mM	Initial gms.	Loss after 4 days on basal diet gms.	Change after 18 days on supple-ments per cent*	Average daily basal food con-sumption in supplement period gms.
3	A					120.8	24.9	—20.2	1.8
8	AA				0.089	116.5	24.7	—20.9	1.9
15	B	0.067				109.5	22.9	— 6.9	2.8
20	BB	0.067			0.089	109.0	21.5	— 3.1	3.1
18	C		0.034			111.5	26.5	—15.2	1.8
16	CC		0.034		0.089	110.0	23.0	—18.4	1.7
21	D			0.034		114.5	29.0	— 5.7	3.3
23	DD			0.034	0.089	105.5	24.5	— 9.0	2.3
26	F	0.134				119.8	25.3	+ 4.2	3.5
22	FF	0.134			0.089	117.1	28.4	+ 6.8	3.6
25	G	0.067	0.034			117.0	27.5	+ 5.8	3.2
27	GG	0.067	0.034		0.089	122.0	28.5	+ 5.8	3.3
6	H	0.067		0.034		121.0	23.4	+12.7	4.5
11	HH	0.067		0.034	0.089	116.5	22.6	+12.9	4.2

*Of weight at beginning of supplement period.

the former two is the more immediate precursor of cystine. Single control rats received no sulfur-containing supplements and supplements of methionine, cystine and homocystine, respectively. A duplicate series received choline in addition to the amino acids. The amounts administered, together with the effects on growth and average daily basal food consumption[2] are given in Table 2.

As was anticipated, cystine supplements (*C* and *CC*) produced no significant response. However, it was unexpected to find that homocystine alone produced substantially the same growth effect as methionine (*B* and *D*) and that supplementation with choline did not improve the response to homocystine. The results of the *F*, *G*, and *H* pairs suggest that a combination of equal parts of methionine and homocystine is superior, at the level fed, to an equivalent amount of methionine only, or to a combination of methionine and cystine. However, before these latter observations could be expanded it seemed essential to investigate the significance of the responses noted when homocystine was the only carrier of organic sulfur.

For further exploratory purposes animals *A* and *AA*, which had no amino acid supplements, were given, after the end of the 18-day period recorded in Table 2, 0.40 mM homocystine daily, while the daily choline supplement of *AA* was doubled (0.178 mM). *AA* responded with an average daily gain[3] during the first 12 days of 2.79 gms. and an a.d.b.f.c. of 4.3 gms. Choline was now withheld, but growth continued during the next 13 days at a moderate rate, with an a.d.g. of 0.62 gms. and an a.d.b.f.c. of 3.7 gms. In *A* the change to homocystine likewise resulted in an immediate change of trend with an a.d.g. during the first 8 days of 1.31 gms. and an a.d.b.f.c. of 2.5 gms. From the 9th to the 13th day, however, the animal failed to eat any basal food, and on the 10th and 11th day administration of the homocystine supplement was impossible on account of the prostration of the animal. From the 12th day onward a daily supplement of 0.178 mM choline was given and homocystine administration was resumed. After having decreased 16.0 gms. in weight from the 8th to the 14th day, the animal started again to eat, and by the 18th day had regained the 16 grams lost.

In summarizing the first experiment, it may be stated that in the

[2] a.d.b.f.c.
[3] a.d.g.

three instances in which homocystine had been the only supplement (*D*, *A* following the 19th day and *AA* after the 31st day) there was evidence of its utilization. In addition, rat *A* showed a peculiar illness-response, the significance of which was unexplained.

2. *Second Series*

In view of these findings, a new series of animals was devoted solely to exploring the nature of the growth effect of homocystine with our experimental material and under our laboratory conditions. Three groups (*A*, *B*, and *C*) of six animals each were given, after

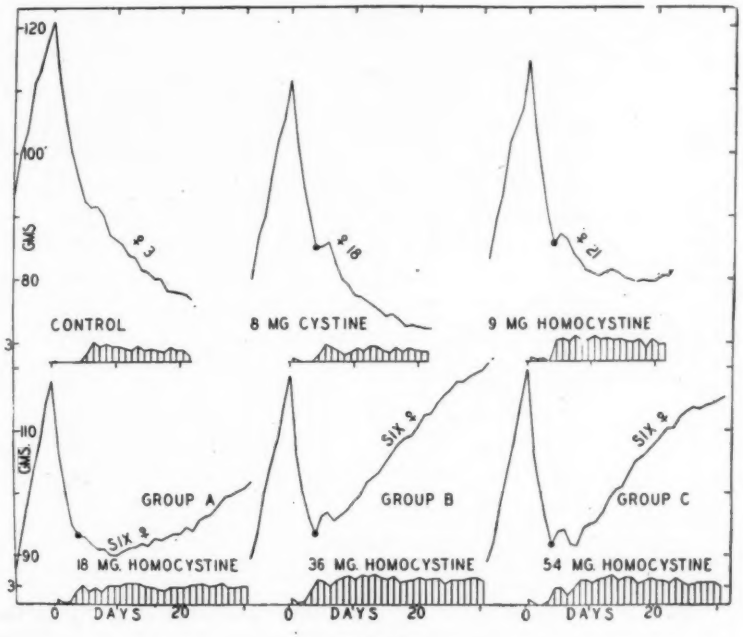

FIGURE 1

DAILY RECORDS OF ANIMAL WEIGHTS AND BASAL FOOD CONSUMPTION

On each weight plot the peak denotes the change from the normal colony diet to the experimental basal diet. A circle denotes the beginning of the administration of supplements. The shaded area shows the consumption of basal food, plotted at the mid-point of each 24-hour period.

the same preliminary treatment as in the first series, daily supplements of 18 mg., 36 mg., and 54 mg. respectively, of homocystine. The three lower graphs of Figure 1 describe the initial phase of this series in terms of group averages. The figure also includes three individual animals of the first series which seem of interest for comparison. For the first 20 days on the homocystine supplements every animal with the exception of one of the *A* group (*A*19) grew. Growth and food consumption during the first 20 days on the homocystine supplements, together with data on the behavior of the individual animals before they were assigned to the three groups, are shown in Table 3.

TABLE 3

RESULTS OF SECOND EXPERIMENTAL SERIES DURING FIRST 24 DAYS

Animal number	Initial gms.	Weight of animals Loss after 4 days on basal diet gms.	Change after 20 days on supplements Per cent*	Average daily basal food consumption in second 10 days† of supplement period
A 7	119.3	24.3	+ 8.4	3.0
A 4	120.9	26.4	+ 8.1	3.2
A21	110.0	21.4	+ 3.8	2.7
A10	115.5	22.5	+ 3.2	3.0
A18	121.0	28.7	+ 3.0	2.7
A19	122.6	27.6	— 6.0	1.9
B 9	116.0	27.9	+32.1	4.4
B11	118.2	22.4	+26.0	4.5
B 6	122.5	27.5	+25.3	4.5
B 2	118.5	23.5	+22.3	4.2
B 5	118.5	21.4	+20.8	4.0
B20	119.0	30.4	+17.9	3.2
C13	125.2	31.5	+32.0	5.0
C 8	122.0	27.7	+28.0	4.3
C12	125.0	32.6	+25.9	3.9
C 1	118.2	23.4	+21.9	3.6
C22	110.5	27.3	+18.0	3.9
C 3	117.0	25.7	+ 7.1	3.3

*Of weight at beginning of supplement period.
†During the first 10 days the animals were kept in pairs.

Figure 2 shows that after about 20 days, while the majority of the animals continue to grow steadily, a few individuals undergo a sudden change. Food consumption declines, and, as a rule, the animal ceases to eat on the third day. At the same time the weight drops rapidly, at the rate of three grams per day or more. In order to illustrate in more detail this phenomenon, which has proved to be a charac-

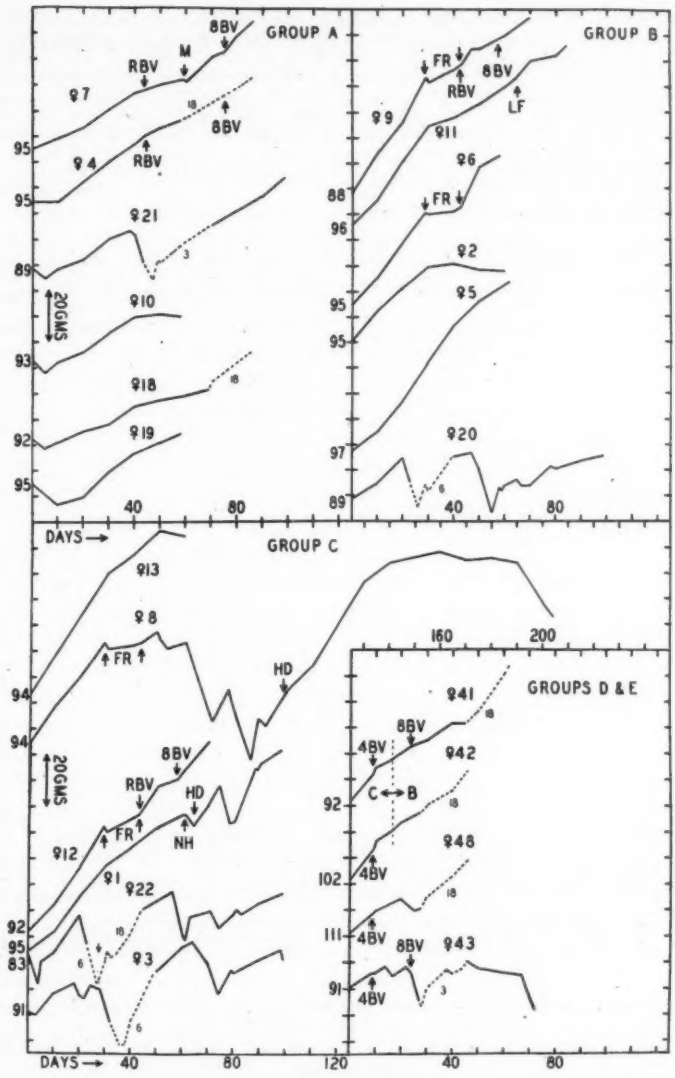

FIGURE 2

GROWTH CURVES OF THE ANIMALS OF THE SECOND EXPERIMENTAL SERIES

Weights are plotted here in 10-day intervals, except (*a*) when a change in the diet

was made, or (*b*) when there were seemingly significant changes in slope (animals *A*21, *B*20, *C*1, *C*3, *C*8, *C*22, *D*43, *D*48). Within each group the individuals are arranged in the order of their net gain during the first 20 days. Symbols: - - - - choline was given (mg. per day in small numerals); RBV, ryzamin B combination; 8BV, eight B-vitamin combination; 4BV, four B-vitamin combination (13); M, 40 mg. dl-methionine per day (in place of homocystine); LF, low fat diet (5% corn oil, instead of 30%); HD, homocystine (1.25%) in the diet; NH, no homocystine; C←|→B, as a vehicle for the supplements Crisco was used before, and butter (as in all other animals) after, the period marked.

teristic occurrence in our experiments, the day-to-day weight and food records of three animals thus affected are shown in Figure 3.

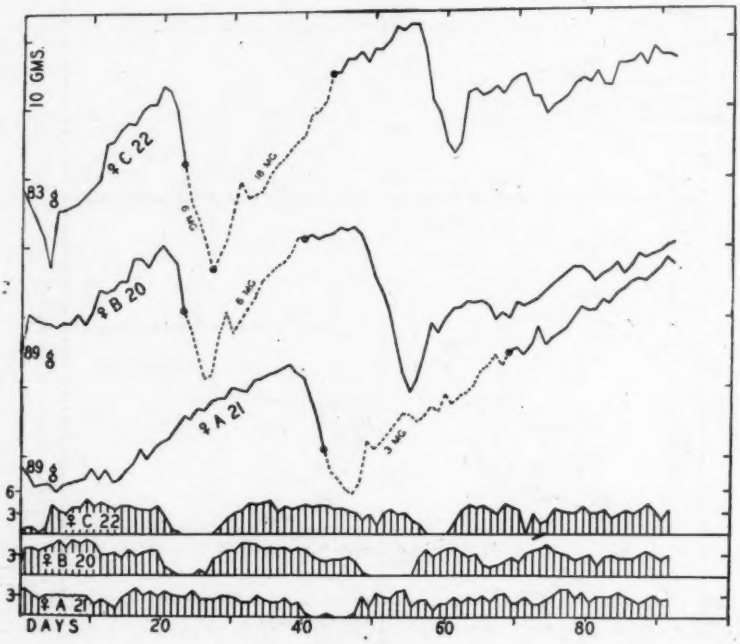

FIGURE 3

DAILY GROWTH AND FOOD CONSUMPTION RECORD OF THREE ANIMALS SHOWING CHARACTERISTIC GROWTH INTERRUPTIONS

Dash lines denote periods of choline (daily amount indicated by small numerals) supplementation.

The characteristic symptoms, which differ somewhat in intensity from case to case, are emaciation, a hunched posture, and listlessness verging on prostration, although as a rule the animal continues to move about feebly. In all severe cases a red deposit, presumably of porphyrin (19, 20) developed on nostrils and paws, and the urine became thick and dark brown, causing extensive staining of the lower abdominal fur. After animals *B*20 and *C*22 had lost 9 and 11 grams of weight, respectively, in three days, they were given daily supplements of 6 mg. choline in an attempt to relieve these symptoms. However, the animals continued to lose weight at an undiminished rate for several days. In the case of *C*22 the choline supplement was then tripled; this was followed by a prompt reversal of the weight trend, although the animal was so weak during the first two days on the larger choline allowance that it could not be forced to eat more than one-half of its regular homocystine supplement. However, animal *B*20, as well as *C*3, showed substantially the same recovery picture with the benefit of only 6 mg. of choline, while in the case of *A*21, where a similar reaction set in on the 39th day, recovery took place on only 3 mg. choline daily. In every case all symptoms of illness disappear within a few days after eating is resumed.

When these animals had exceeded their former maximum weights the choline supplements were withheld. Growth continued undiminished in the case of *A*21 for a further 30 days, at which time the animal was autopsied. However, *B*20, *C*22, and *C*3 developed new breaks 9, 13, and 16 days, respectively, after withdrawal of choline, which in their symptoms were indistinguishable from the initial breaks. Although no choline was given in these instances, extent and duration of the three secondary declines were substantially the same (average loss 21 grams in 8 days) as in the primary declines (average loss 22 grams in 8 days). Subsequent observations (*C*1, *C*8) confirm the impression that the animals are capable of passing through this crisis successfully without receiving choline supplements at any time. The example of animal *A*18 shows, on the other hand, that a diminished growth rate responds favorably to supplementation with choline.

Since the few animals that showed the acute breaks in their growth curve seemed to be recruited from the poorer eaters (and growers) of their respective groups, the food intake of four rats was restricted

to 3 grams a day, to see if a break might be elicited thereby. On the other hand, in view of the finding of Griffith (21) that lower food consumption was correlated in his series of choline-deficient rats with less severe renal lesions, one might also reason that restriction of the daily food intake should prevent these typical growth crises. The records of *B*6, *B*9, and *C*8, *C*12 (Figures 2 and 4) by compari-

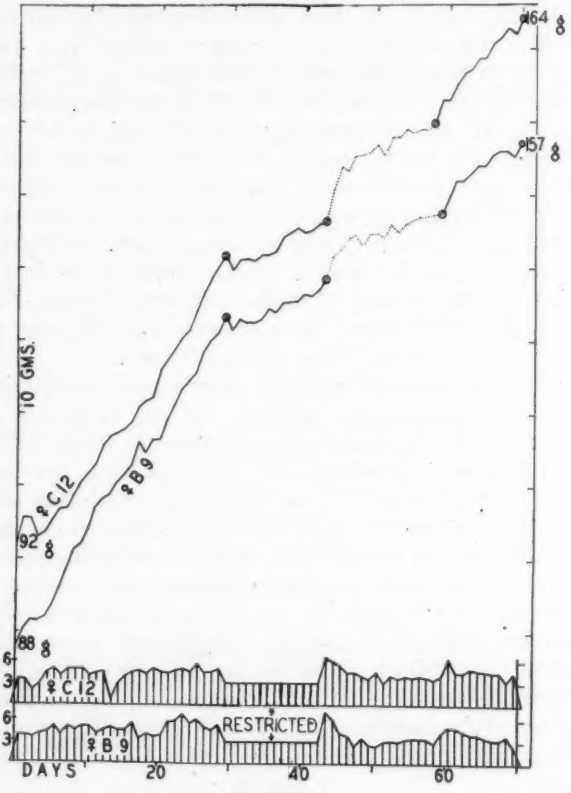

FIGURE 4

DAILY GROWTH AND FOOD CONSUMPTION RECORD OF TWO ANIMALS, ILLUSTRATING THE EFFECT OF RESTRICTED FOOD INTAKE AND CHANGE IN VITAMIN REGIMEN

Dotted lines indicate period during which the eight-vitamin combination was replaced by the ryzamin combination.

son with those of the similar but unrestricted pairs *B*2, *B*11, and *C*1, *C*13, do not support the former possibility. The restricted diet was not continued long enough to secure evidence in regard to the second alternative.

In order to see if the ability of these animals to grow in the absence of a dietary source of "labile" methyl groups is related to their vitamin regimen, our supplement of eight B-vitamins was replaced in four animals (*A*4, *A*7, *B*9, and *C*12) by the modified ryzamin B combination which du Vigneaud *et al.* (13) employed in the pioneer work on transmethylation. During 15 days of this treatment the a.d.g. of *A*4 was 0.4 grams compared with 0.7 grams in the preceding 15 days on the full vitamin complement, and compared with 0.6 grams on the subsequent 15 days when 18 mg. choline daily were given with the ryzamin combination. Return to the full vitamins, with continuation of the choline supplement, resulted in a.d.g. of 0.7 grams during the next 11 days. In *A*7 during 15 days on ryzamin the a.d.g. was 0.3 grams compared with 0.5 grams in the preceding 15 days' period. In this instance the regular supplement of 18 mg. homocystine was next replaced by 40 mg. methionine daily, and during 15 days the a.d.g. was 0.55 grams. Return to the full vitamins, with continuation of this methionine supplement, increased the rate to 1.1 grams during the next 11 days. In the cases of *B*9 and *C*12, the effect of the change in vitamin intake is shown in full in Figure 4. After an initial spurt of excessive eating accompanying the release from the restricted food intake, a moderate growth rate develops on the ryzamin combination, and a significant increase ensues upon return to the full vitamin supply.

The growth curve of animal *B*11 shows that growth is not critically dependent on the dietary fat level. In animal *C*1 withholding of the homocystine supplement is followed promptly by cessation of growth, which is vigorously resumed when a dietary mixture containing 1.25 per cent of homocystine is given without butter supplements. The same change was instituted in animal *C*8 after it had been on the butter-homocystine supplements for about 100 days. The animal continued to grow, and was killed for autopsy after more than 200 days on the choline-free diet.

Figure 2 includes the growth record of four additional animals (*D*43, *D*48, and *E*41, *E*42). All were given 36 mg. homocystine daily,

but two received it in the usual mixture with butter, while in the other two butter was replaced by hydrogenated vegetable oil (Crisco) in order to ascertain if possibly some ingredient of the butter might be involved in enabling the animals to grow. The results, together with those of *C*1 and *C*8, show that the butter is not a significant factor, and the four animals were further utilized for some exploratory observations on the rôle of the vitamin supplements, the details of which are shown in Figure 2.

This second series of experiments seems to indicate that there is a definite growth response when homocystine alone is fed with a choline-methionine-free basal diet supplemented with the eight B vitamins, and this response is related to the amount of homocystine fed and also to the ability of the individual rat to metabolize it. After 20 days, about one-half of the animals continued to increase in weight for two months or more (*A*4, *A*7, *A*19, *B*5, *B*6, *B*9, *B*11, *C*1, *C*12, *E*41, *E*42), while the growth of approximately one-sixth leveled off or showed a slow decline (*A*10, *A*18, *B*2, *C*13), and the remaining third went through the characteristic drop in weight followed by recovery. The fact that growth was retarded when the ryzamin B combination was fed to four rats, suggested that the presence of some of the B vitamins, supposedly very low or absent in the ryzamin combination, aided the response to homocystine, somehow making possible its methylation without the benefit of choline. In order to test this hypothesis, a third experiment was undertaken.

3. *Third Series*

This series of experiments was planned to compare the growth response to homocystine when our eight-vitamin combination was fed on the one hand, and the ryzamin combination on the other. In order to be sure that the method of feeding the homocystine would not complicate the results, it was decided to give it in our usual manner as a supplement, 36 mgs. a day, and also as 1.25 per cent of the basal diet, i.e., by the technique of du Vigneaud. Because, according to the results of the second series, a larger supplement does not improve growth but tends to increase the frequency of the observed acute illness, 36 mgs. were given. On an average food consumption of three grams per day, the amount of homocystine received would be approximately equal in the two groups.

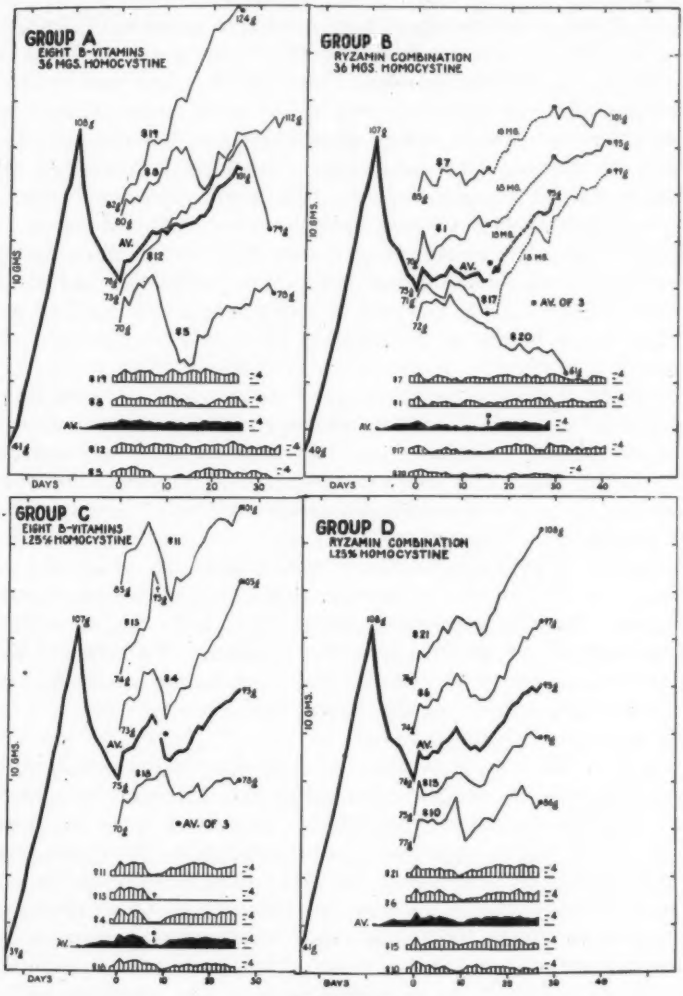

FIGURE 5

DAILY GROWTH AND FOOD CONSUMPTION RECORD OF THIRD EXPERIMENTAL SERIES
Dash lines denote periods of choline (daily amount indicated by small numerals) supplementation.

As shown in Figure 5, four groups of rats were used, two of which (*A* and *B*) were fed homocystine as a separate supplement, while the other two (*C* and *D*) got it in the diet. One group of each pair (*A* and *C*) received the previously used basal ration and the daily supplement of eight vitamins, while in the other group of each pair (*B* and *D*) the ryzamin combination was given and the fat soluble vitamins of the basal diet were slightly modified in conformity with Chandler and du Vigneaud[4] (13). The graphs (Figure 5) include the group averages for 15 days' growth on our standard colony diet and the ensuing loss of weight and food consumption when the animals were placed on the basal diet. This preliminary period was extended to nine days in the present series in order to establish more uniform eating habits in the animals before the supplements were given.

Group A. Two of these rats showed the previously described drop in weight which was followed by recovery without choline, while the other two showed sustained growth. The weight losses occurred earlier in this group than in the corresponding group *B* of the second series, but the a.d.g. (percentage) during the first 20 days is strikingly similar in these two groups.

Group B. Two of the rats lost weight after an initial rise, two continued to grow slowly, the average of the four showing no increase in weight. When choline was given to three of the rats as indicated by the broken lines, improved growth resulted. When two of these animals were deprived of choline, the slopes leveled off again. The basal food consumption of this group was less than that of the *A* group even when choline was fed.

Group C. Two rats grew well, but showed a typical drop in weight from which recovery resulted after eating had slackened for a period. One animal, *C*13, died on the 8th day, perhaps from excess homocystine intake due to high basal food consumption, which was higher for this group (average 46 mg. per day) than for any of the other groups. This death was the only one encountered in all the experiments. Before death the animal drank much water, causing a large increase in weight, out of proportion to the food intake, and autopsy showed excess fluid in the peritoneal cavity. The fourth rat did not

[4]In place of carotene and methylnapthoquinone (*cf.* footnote 1) 833 mgs. of halibut liver oil (60,000 USP units of vitamin A per gram, S. B. Penick & Co.) were used.

grow well at any time and its basal food consumption remained low.

Group D. All four of these rats grew and showed a drop in weight followed by recovery, but these drops were not as severe as those in groups *A* and *C* and were associated with only slight decreases in food consumption. The latter was almost identical with that of group *A* and the average homocystine intake was not much above that of *A* and *B*, about 40 mg. a day.

As measured by the averages, the net increases in weight of the four groups are approximately equal in spite of the individual variations within the groups. However, the increase was not attained by the B group until choline was given in addition to homocystine.

F. Autopsies

Animals to be autopsied were chloroformed and promptly dissected. Organs were weighed and in many cases sections were prepared for histological examination. On a number of animals of the third series liver lipids were determined by the method described in detail in the accompanying paper (22). In the first series only liver weights were recorded. The incompleteness of the data in the second and third series is due mainly to the fact that a number of the bodies were used for special investigations, such as determination of methionine, cystine, and total labile methyl content.

Table 4 shows that in all pairs of the first series the absence of dietary choline has resulted in an increased liver weight, presumably reflecting fat accumulation. The difference is more pronounced, as a whole, when active growth has taken place at the expense of cystine or homocystine; but in the *D, DD* pair the difference is marked, even though the "choline-free" partner did not grow.

Tables 5 and 6 summarize the data from the second and third series. It is to be noted that all of these animals show evidence of having livers of high fat content, although to varying extents. The nature of the pathological changes encountered is similar to those described by du Vigneaud *et al.* (23) resulting from the administration of homocystine. In the second series the more severe liver changes involving cell injury were absent in all those animals, and only in those, that had received choline or methionine at some time, even though the lipotropic supplement may have been discontinued as far back as 60 days before death (*B*20). However, the extent of fatty infiltration tends

TABLE 4

AUTOPSY DATA ON ANIMALS OF FIRST EXPERIMENTAL SERIES

No choline fed				11 mg. choline per day				
Rat No.	Days on experiment	Body weight gms.	Liver weight per cent of body weight	Rat No.	Days on experiment	Body weight gms.	Liver weight per cent of body weight	Supplements
A*	37	88	5.8	AA†	43	114	4.1	homocystine‡
B	36	93	4.5	BB	36	92	2.5	methionine + cystine§
C	18	72	3.4	CC	18	71	3.0	cystine
D	43	74	4.6	DD	36	88	2.8	homocystine + cystine§
F	36	120	3.2	FF	36	117	3.0	methionine‖
G	18	95	4.1	GG	18	99	3.2	methionine + cystine
H	18	110	3.8	HH	18	106	3.4	methionine + homocystine

*During the last 7 days 22 mg. choline was given.

†The choline supplement was doubled after 18 days and completely withheld during the last 13 days.

‡For details cf. text.

§Subsequent to the periods covered in Table 2, 20 mg. of cystine was added to the methionine supplement for an additional 18 days.

‖Subsequent to the periods covered in Table 2, the methionine supplement was increased from 20 to 60 mg. for an additional 18 days.

to be greater, the longer the choline-free period preceding death. Among the cases of liver injury it does not seem possible to relate the degree of damage to the relative growth capacity.

The quantitative data on liver fat, available in the third series, show that within each of the three groups which received no lipotropic supplements, fat deposition in the liver runs parallel with the relative growth rates, while in the *B* group where choline was given (and apparently needed to establish growth) the relation is inverse, the weight-losing animal (*B*20) showing the highest liver fat figures, while the best grower (and choline beneficiary) has the lowest. It seems difficult to reconcile the evidence of rat *B*20 with the conclusion of Handler (24) that development of the fatty liver characteristic of choline deficiency depends on the growth of the animal.

The liver fat values in the *D* group seem to be significantly lower than in the *C* group. Net growth of the two groups, which differ in their vitamin regimen, is substantially identical; however, food consumption is higher and the growth crises are more severe in the *C* group.

In the examination of the kidneys two types of injuries were predominantly encountered, viz., evidence of present or past hemorrhages, and evidence of active chronic nephrosis involving mainly the proximal convoluted tubules. Among the 14 autopsied animals which had grown substantially without having received choline or methionine at any time, the kidneys were normal in two cases, evidence of healed hemorrhages were found in four, six showed evidence of recent hemorrhages, associated in all but one case with nephrosis, and two showed nephrosis only. Casts were seen in four of these cases. In only one case (*D*15), could the hemorrhages be described as massive in character. Among the four animals of the second series which were severely handicapped by recurrent growth interruptions (*B*20, *C*22, *C*3, *D*43) the kidneys were characterized in three cases by evidence of healed hemorrhages, while the fourth showed nephrotic symptoms. That the comparatively healthy kidney picture in the former three cases is connected with the temporary administration of choline (which seems to have a protracted favorable effect on the liver, cf. p. 75) can only be surmised. The fourth of these animals, in which nephrosis was encountered, was autopsied while in the trough

TABLE 5

AUTOPSY DATA ON ANIMALS OF SECOND SERIES

Rat No.	Weight of liver. % of body weight	Weight of kidneys. % of body weight	Histopathology of liver: Fat infiltration without visible cell damage: Limited, localized	Histopathology of liver: Fat infiltration without visible cell damage: Occupying more than half of tissue	Histopathology of liver: Fat infiltration throughout, fatty degeneration of cells	Histopathology of kidneys**: Cortical hemorrhages: Healed, connective tissue invasions	Histopathology of kidneys**: Cortical hemorrhages: Present or recent (blood cells)	Histopathology of kidneys**: Nephrosis of proximate convoluted tubules	Histopathology of kidneys**: Glomerular hypertrophy	Histopathology of kidneys**: Casts
A 7	3.6	0.72	+					normal		
A 4	3.7	0.77	+					normal		
A21	6.3	0.77		+				normal		
A10	6.1	0.89			+	+		+		
A18	4.9	0.85	+					normal		
A19	5.7	0.95			+			normal		
B 9	5.5	0.80			+			+		+
B 6	4.8	0.76			+		+	+		
B 2	5.7	0.98			+		+*	+		
B20	6.5	0.93		+		+	+			
C13	4.6	0.87			+	+				
C12	5.9	1.00			+			+		+
C22	6.2	0.90		+		+				
C 3	6.2	0.97		+		+			+	+
E41	4.3	0.76	+					normal		
E42	3.6	0.82	+					normal		
D48	3.9	0.80	+					++		+
D43	5.4	1.03		+				++	+	+

*Confined to capsule.

**The word "normal," wherever used, refers to all five columns.

TABLE 6

AUTOPSY DATA ON ANIMALS OF THIRD SERIES

Rat No.	Weight of liver. % of body weight	Weight of kidneys. % of body weight	Liver fat		Histopathology of kidneys**				
			Per cent of liver weight	Per cent of body weight	Cortical hemorrhages: Healed, connective tissue invasions	Cortical hemorrhages: Present or recent (blood cells)	Nephrosis of proximate convoluted tubules	Glomerular hypertrophy	Casts
A19	8.7	0.90	32.2	2.8			normal		
A 8	6.9		30.9	2.1					
A12†	6.8								
A 5	7.4		35.1	2.6					
B 7‡	4.1	1.02					normal		
B 1	5.1		27.1	1.4					
B17	5.5		15.0	0.8					
B20	5.6		28.7	1.6					
C11	6.1	0.99	28.7	1.8		++	+++§		++
C13‖	6.7	1.97				++	+++	+	
C 4	7.9	1.11	32.5	2.6		+	++		+
C18	5.8	1.40	30.7	1.8		+	++		+
D21	6.1	0.96	29.3	1.8	+				
D 6	6.1	1.13	25.9	1.6	+				
D15	5.5	1.16	25.5	1.4	+++	++++			
D10	4.8	0.95	21.7	1.0	++				

†This animal was continued for 40 days longer than shown in Figure 5. Homocystine (1-1.25%) was part of diet mixture during this period. The final weight was 144 gms.

‡This animal was continued for 32 days longer than shown in Figure 5. Some exploratory modifications in the supplement of B-vitamins were tried during this time. The final weight was 107 gms.

§Collecting tubules were also involved.

‖This animal showed on autopsy edema of the tissues and ascites (cf. p. 74).

**See footnote of Table 5.

of its second crisis. The histological changes were not more severe than among animals in other phases of their growth curves. Nephrotic symptoms were equally or more prominent in the animals of the *C* group of the third series, which were autopsied within 15 days after they had emerged from crises of varying severity, and all showed evidence of recent hemorrhages. While the picture of nephrosis thus seems to link itself with the acute growth crises, with the implication that during a recovery period of several weeks these symptoms may disappear, this relation is hardly adequate to explain the swift course of events revealed by the weight and food charts. A rapidity both in onset and recuperation would be required which is difficult to reconcile with the impression gained from the study of the slides, of a chronic nephrosis arising by the gradual accumulation of slight injuries. A more likely hypothesis would assume that during the few days of fasting certain enzyme systems of the liver may be freed sufficiently, as a result of the burning of accumulated lipid or related substances, to permit resumption of impaired metabolic functions. In that event the kidney damage would be the result rather than the cause of the metabolic disturbances revealed by the growth crises.

G. Discussion

It will be assumed for the purposes of this discussion that with regard to the absence of donors of labile methyl groups, our basal diet is substantially identical with that of du Vigneaud *et al.* (13). This premise is based on the similarity in composition, on the origin of the components, and on our own analytical results. Many of our rats have grown at rates that compare well with those obtained on synthetic diets containing methionine, and one might surmise that an essential minimum of synthetic tasks, including those requiring labile methyl groups,[5] is being performed in spite of the absence of dietary

[5]The minimal amount of such groups involved in normal growth may be roughly estimated, as follows: Available data on the nitrogen content of rats (25), the non-protein fraction of the total nitrogen (26) and the amino-acid constitution of tissue proteins (27) yield a tentative figure of approximately 6 mg. for the amount of methionine normally being incorporated into body proteins per gram of gain in body weight. To this one may add 0.9 mg. of choline (9) and 2.3 mg. of creatine (25) per gram of gain in weight. Daily excretion of urinary creatine and creatinine would add another 4 mg. of creatine (28). On the conservative basis of a daily gain of one gram we thus get a daily requirement of 0.11 milliequivalent of labile methyl, corresponding to 4.4 mg. of choline if its three methyl groups are completely utilized, or 16 mg. of methionine.

methyl donors. However, this question has been made the object of separate investigations the first of which appears in the adjoining paper (22). The pathological findings do not appear to conflict with the view that growth in our rats is essentially normal. The regular appearance of fatty livers is not surprising since in experiments of prior authors (13, 29) involving methionine- or choline-containing diets, homocystine and homocysteine, even in amounts much smaller than those given our animals, had displayed antilipotropic action resembling that of cystine. It has likewise been shown previously that development of fatty livers does not preclude growth but is even favored by the increased metabolism associated with growth, and may be prevented when net growth is inhibited by the withholding of any of a number of dietary essentials (21, 24).

The relation of kidney damage to the ability to grow seems different from that of liver involvement. Griffith's data (21) show that the level of dietary choline necessary for the prevention of kidney damage is substantially lower than that required for the abolishment of excess liver fat, and that the development of kidney lesions is accompanied by loss of appetite and weight (30). At the risk of oversimplification, it may be said that with increasing contraction of the methyl supply, symptoms develop in the following order: first, fattiness of the liver; second, cessation of growth; and, third, kidney damage; with the possible corollary that a certain degree of functional integrity of the kidneys is indispensable for growth, and that a metabolic situation permitting a minimum growth is a prerequisite for the accumulation of liver fat. One may further view the intensity and frequency of the nephrotoxic symptoms as the resultant of the action of lipotropic and anti-lipotropic factors upon the biochemical background of the individual animal. The sensitivity of the latter to anti-lipotropic influences depends on age, sex, and strain, but, in addition, varies greatly, for unknown reasons, among individuals where these factors are identical (31). The differential behavior of our animals appears to be the result of such variability, since with increasing levels of the anti-lipotropic factor (homocystine), the frequency of kidney disturbances, as reflected in the appearance of the urine and the animal as a whole, and in the growth and food consumption curves, increases.

The evidence obtained in the autopsies supports the view that a

mild degree of kidney hemorrhage like that described by Griffith (21) and Christensen (32) is at least one important factor in those of our animals which were incapable of sustained growth on the 8-vitamin and homocystine rations. However, despite the general resemblance of our growth disturbances to those of Griffith, there is a notable difference in that no deaths occurred among the 22 animals of our second series, which covered average experimental periods of 80 days, while in Griffith's rats (30), which were males, and younger than ours during their critical period, there was a mortality of 24 per cent during 30 days on a 15 per cent casein, 0.3 per cent cystine diet. That average extent and duration of the growth interruptions recorded by Griffith (rf. 30, Figure 1, Curve 6) are much smaller than ours may be the result of the elimination by death of the more severely affected animals. Growth interruption patterns that resemble ours more strikingly were encountered by Cox, Smythe, and Fishback (33) in a study of the nephropathogenic action of cystine.

The fact that our animals grew without a known source of dietary N- or S-bound methyl groups is not without precedent. Du Vigneaud *et al.* (2) called attention to isolated animals which grew as much as 0.4 gms. per day for eight weeks on their methyl-free homocystine diet and considered the possibility that intestinal processes analogous to those known as refection (34) may be involved. Previously Brand (35) had recorded daily growth rates of 1.1 and 1.7 gms. induced by homocystine in the presence of milk concentrate and yeast supplements which probably (2, 11, 34) supplied at the most 2 mg. each of methionine and choline per day. Growth in response to homocystine which can hardly be accounted for by dietary methyl donors has also been obtained by White and Beach (36). While addition of cystine to their arachin diet was ineffective, the effect of homocystine and methionine was positive and similar in magnitude. Their daily vitamin supplement of ryzamin B and Eli Lilly dry liver extract presumably contained less than 2 mg. of choline.[6]

In explanation of these recorded cases, and our own, various possibilities may be considered. If one postulates the existence of labile methyl groups in unknown combinations, these could be present either

[6]For the choline content of ryzamin B, *cf.* section "Diet"; the liver extract contains 0.8-1.2 per cent of choline, according to data supplied by Dr. A. Lee Caldwell of the Lilly Research Laboratories, whom we wish to thank here.

in the diet, or they could exist in the animals as depots built up from the pre-experimental diet. On the other hand, intestinal absorption, or coprophagy may furnish the animal with labile methyl groups originating from bacterial metabolism; or there may be vitamin factors, of either dietary or intestinal origin, the presence of which may enable the animal to compensate for the absence of dietary methyl donors by biosynthetic means of its own or of its intestinal bacteria. Reports on the strong lipotropic action of injected 2-methyl, 1,4-naphthoquinone (vitamin K) (37) and a related effect of N, N-dimethyl-p-aminoazobenzene (butter yellow) (38) show that the first of these possibilities cannot be ruled out. However, for the explanation of the difference in the dietary methyl requirements of our animals and those of du Vigneaud this would not seem to be relevant since we assume that the respective diets are fundamentally identical. The second possibility would have to be explored if future work should show that the ability to grow without dietary methyl donors is a function of the pre-experimental diet. Concerning the third possibility, an investigation, to be published separately, is at present under way. Meanwhile, some grounds seem to exist for a closer consideration of the last-named alternative. Comparison of groups *A* and *B* of our third series (Figure 5) clearly shows a situation where the same net effect on growth (and food consumption) can be produced by feeding either choline or certain additional vitamins. If growth is restricted here by limitation of the methyl supply, we have an instance of enlargement of that supply by the introduction of additional dietary vitamins. Support for the theory that a vitamin-like factor is involved in the regulation of the methyl supply may also be seen in the work of Entenman and Chaikoff (39) if we concede that lipotropism of the choline-deficiency fatty liver involves a drain on that supply. Their heat-labile pancreas fraction AR showed a lipotropic effect at least 20 times as high as that of its maximum possible choline content.

Any hypothesis invoking vitamins in explanation of our results must take into account the evidence obtained in Groups III *C* and *D* (Figure 5). The experimental nutrition of Group *D* is a reproduction of that used by du Vigneaud, and the results show that our animals grow as well on the ryzamin combination as on the more complete eight-vitamin combination (Group *C*), although there are

characteristic differences in the growth patterns. Recent developments (40) have made it clear that the effective vitamin supply is by no means identical with the dietary supply, as the former includes important contributions originating from the intestinal flora. The nature of the pre-experimental diet will undoubtedly be an important determinant of the flora prevailing at the beginning of the experiments, and perhaps our rich pre-experimental diet is particularly favorable to bacterial synthesizers of certain vitamin factors involved in methylation. The variable extent to which this pre-experimental intestinal flora might carry over into the experimental periods could then explain the differences in the ability of individual animals to utilize homocystine. If we further assume, in concord with Elman's (41) findings on this point, that simultaneous ingestion of dietary components (as in Groups III *C* and *D*) insures more complete utilization than when one component (homocystine) is given in a single separate feeding (as in Groups III *A* and *B*), the better utilization of limited vitamin resources (Groups III *B* and *D*) in the former mode of feeding appears likewise reasonable. In the same connection it should be recalled that the results of our second series (Figure 2) indicate that the amount of homocystine ingested is an important causative factor in the precipitation of the growth crises. The data of the third series suggest that also in this aspect homocystine is more effective when fed in the diet (Group III *C*, average daily homocystine ingestion before the first drop in weight begins, 54 mg.; average duration of this period, 7 days) than when fed separately (Group II *C*, 54 mg., and more than 40 days, respectively).

The association of the so-called "porphyrin nose" with our growth derangements (cf. p. 69) has undoubtedly its vitamin connotation. The paradox presented by the appearance of this pantothenic acid deficiency symptom (19) in rats receiving a generous daily supplement of that vitamin, can be resolved if the suggestion (42) is accepted that the utilization of pantothenic acid depends on the availability of folic acid and biotin. The fact that the latter is included in our daily supplement would explain the complete absence of the spectacled-eye condition (40) in our animals. However, a disturbance of the intestinal synthesis of folic acid brought about somehow by the homocystine, non-choline diet in some individuals[7] may well be

[7]The irregular appearance of acute symptoms seems to be characteristic for deficiencies resulting from failures of intestinal synthesis [*cf.* (43), (44)].

responsible for the association of the porphyrin symptoms with the acute growth failures. This theory would also account for the above-mentioned differences in the growth patterns of Groups III *C* and *D*. Welch and Wright (45) observed a distinct folic acid effect with a rice-polish concentrate, i.e., the type of vitamin product represented by ryzamin B. Thus the animals getting the ryzamin combination, while receiving substantially less of most of the known factors than the animals which get the eight-vitamin supplement (see Table 7),

TABLE 7

VITAMINS CONTAINED IN DAILY SUPPLEMENTS, ACCORDING TO AVAILABLE DATA

	Ryzamin combination			Eight-vitamin supplement
	Present in 25 mg. of ryzamin B*	Added	Total	
	γ	γ	γ	γ
Thiamin	6	20	26	40
Riboflavin	0.6	20	21	40
Nicotinic acid		20		500
Pyridoxin	2.5	0	2.5	40
Pantothenic acid	10	0	10	200
p-Aminobenzoic acid	†‡	0		500
Inositol	†	0		500
Biotin	†	0		0.25
Folic acid	§	0		0
Choline	200‖	0		0

*We are indebted to The Burroughs Wellcome Co., U. S. A., for the data on which these figures are based.

†Known to be present "probably in relatively smaller concentrations" (cf. *).

‡1 γ per gram (45).

§A rice polish concentrate as 1 per cent of the diet was found to compensate fully for the inhibition of intestinal folic acid synthesis produced by the presence of 1 per cent succinylsulfathiazole in the diet (44).

‖Cf. p. 61.

would be at an advantage in that they obtain a steady supply of the folic acid factor(s) which would enable them to surmount a temporary failure of intestinal folic acid synthesis with only the minor difficulties revealed in the growth curves of Group III *D*.

This suggested rôle of folic acid in the acute growth failures does not imply that it should also be the decisive factor in the mobilization of methyl groups. Involvement of vitamin K or its isotels[8] in this process appears more likely. The methyl group of 2-methyl-1,

[8]Compounds performing the same function; according to the terminology proposed by R. J. Williams (47).

4-napthoquinone seems to be labile (37), the compound can combine with SH compounds (48) and the amount of K vitamins available may vary considerably with intestinal conditions (44).

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H. Summary

It is shown that sustained growth of rats can occur as a result of the addition of homocystine to synthetic diets devoid of choline, methionine, and other known sources of N- or S-bound methyl groups. In a certain percentage of the animals, varying with the amount of homocystine fed, characteristic and spontaneously reversible growth interruptions appear which have not been observed in animals receiving choline. The relations of these growth responses to different dietary vitamin combinations and to different modes of homocystine administration have been examined. While our rats grew under substantially the same dietary conditions which in the investigations of du Vigneaud did not produce growth unless a methyl donor was supplied, the animals failed to grow when homocystine was given as a separate supplement; but growth could be induced by either supplying choline or a combination of the eight other known members of the B-complex and methylnaphthoquinone. Some implications of these findings have been discussed.

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THE SYNTHESIS OF METHIONINE AND CYSTINE BY RATS ON A CHOLINE-FREE DIET WITH HOMOCYSTINE AS THE ONLY SULFUR-CONTAINING AMINO ACID*

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Since it has been demonstrated in the preceding paper (1) that rats are able to grow on a methionine- and choline-free diet containing homocystine, it becomes of importance to ascertain whether methionine is synthesized in amounts which enable the rats to maintain a normal composition or whether their excess weight is due to enlargement of bony structures and other non-protein constituents.

Three rats were selected for analysis from among the best growers of those used in the preceding investigation. They were *B*5, *C*1, and *B*11 of the second experiment of that series, and are here designated as *A*, *B*, and *C* respectively. Details of the special constituents of their diets are given in Table 1. As may be seen, *A* and *C* received

TABLE 1

AGES AND WEIGHTS OF THREE RATS (*A*, *B*, AND *C*) MAINTAINED ON A CHOLINE-FREE DIET WITH HOMOCYSTINE AS ITS SOLE SULFUR-CONTAINING AMINO ACID AND FOUR CONTROL RATS (*D*, *E*, *F*, AND *G*)

Age (days):							
At start of exper.	40	40	40				
At death	105	138	125	40	39	58	125
Weight (grams):							
Body:							
At start of exper.	118.5	118.2	118.2				
At death	163.6	171.0	165.4	118.2	122.2	162.5	283.5
Gain	45.1	52.8	47.2			47.0	165.3
Carcass	155.2	160.6	155.9	105.1	111.6	149.6	265.2
Liver	7.61	7.48	6.50	6.46	7.42	7.35	10.59
Total carcass	162.8	168.1	162.4	111.6	119.0	157.0	275.8
Total carcass dehydrated and extracted	38.0	37.7	39.5		29.2	39.0	70.8

*Aided by a grant from the Women's Auxiliary of the Lankenau Hospital Research Institute.

36 mg. of homocystine daily while *B* received 54 mg. for 61 days. It then was without amino acid supplements for three days, after which 1.25 per cent of homocystine was incorporated in the basal food for the final 34 days of the experiment. During this latter period the rat consumed a total of 152.5 grams of diet mixture, containing 1.91 grams of homocystine, or an average of 56.1 mg. a day. Details as to their ages and weights of the rats are also incorporated in the table (also see Figure 2 of the preceding paper).

Four additional female rats (*D-G*) were employed as controls. They had been fed on the routine mixed laboratory diet similar to that used for *A*, *B*, and *C* before being placed on the methionine-free diet. Rats *D* and *E* were killed at the age of 40 and 39 days, at which time they were of about the same age and weight as the three preceding rats at the beginning of the experiment. Rat *F* was killed at the age of 48 days. It then weighed approximately the same as the three experimental animals at death. Rat *G* was sacrificed at the age of 125 days, the same age as Rat *C*.

A. Analytical Technique

At the close of the feeding period, each rat was chloroformed and the liver removed immediately, weighed, and placed in dry ice in a mortar. When frozen solid it was ground to a powder. The gut was dissected from the carcass and washed under a tap. The carcass, including the washed gut, was weighed, ground in a meat chopper, reweighed, transferred to a tared round bottom flask and covered with 500 ml. of acetone. The ground liver was transferred separately to a tared 100-ml. centrifuge tube, reweighed and covered with 50 ml. of acetone. Acetone and ether extractions were carried out as described by Rose and Wood (2) and the acetone-ether extracts were used for the determination of liver and carcass fat.[1] The two residues were combined, dried in a vacuum oven according to Rose and Wood, and hydrolyzed for estimation of total nitrogen, amino nitrogen, methionine, and cystine.

1. *Estimation of Fats*

The ether was distilled off from the ether extracts of the carcasses,

[1]The term "carcass fat" employed in the paper, refers to fat extracted from the carcass minus the liver. "Total carcass" designates the carcass including the liver.

after which the accumulated acetone extracts were added and also distilled off. The watery residues, usually about 100 ml. in amount, were concentrated over a steam bath to about 25 ml., transferred to a separatory funnel, extracted about eight times with ether, or until a drop of the ether, dried on a watch glass, indicated that extraction was complete. The filtered ether was again distilled off, and the fatty residues transferred qualitatively to weighing bottles, freed of ether over a warm oven, and the fat dried to constant weight in a desiccator containing oil and calcium chloride. The liver extracts were treated similarly except that correspondingly smaller volumes were employed. The watery residues were discarded after tests for protein, methionine, and cystine had proved negative.

2. *Hydrolysis of Protein*

Each combined carcass and liver was placed in a tared round bottom flask in a vacuum desiccator over $CaCl_2$ and oil, connected to the water pump for 4 to 6 hours, and then let stand for 48 hours until acetone- and ether-free. Hydrolysis was accomplished by a method of Lavine (3): Water equal in weight to that of the extracted residue was added, then twice that amount of 12 *N* HCl by volume. Solution was brought about by standing on the steam bath for 3 hours, after which it was refluxed in an oil bath at 140° C. for 48 hours. To ensure complete hydrolysis, a sample was removed from the first hydrolysate after 4 hours, and again after 8 hours of heating, decolorized, and the methionine and amino acid content determined. A rise of two per cent in each value was found. With the subsequent hydrolysates the reaction was assumed to be complete at the close of the 8-hour period. The solutions were then concentrated in vacuo to a syrup and placed in a vacuum desiccator for 12 hours over NaOH and $CaCl_2$. To the dark brown hydrolysates, 350 to 400 ml. of water were added, the acidity determined, and the normality brought to about 0.4 with NaOH. Decolorization with activated charcoal followed (4), the charcoal being immediately filtered out, the filter paper washed repeatedly with water, and the volume made up to one liter. Total nitrogen, amino nitrogen, methionine, and cystine were determined on the decolorized hydrolysates.

Total nitrogen was determined by micro-Kjeldahl and Nesslerization.

Amino nitrogen was estimated by the Kendrick and Hanke (5) modification of the Van Slyke procedure.

Methionine was estimated by the procedure of Lavine (3). Homocystine, in this determination, behaves, to a certain extent, like methionine, but the liberation of iodine from the iodonium salt is much slower and gives rise to a lagging end point. The methionine determinations gave no evidence of homocystine for the normal or experimental animals.

Cystine was precipitated as the cuprous salt by the technique of Rossouw and Wilken-Jordan (6), recovered as cysteine with H_2S (7) and determined colorimetrically by the Sullivan method. No differentiation between cysteine and cystine was attempted. All values are recorded as cysteine. With this procedure homocystine, also precipitated by cuprous chloride, gives rise to a typical purplish color which disappears on the addition of the hydrosulfite. There was, again, no evidence of homocystine. As an extra precaution, the colorimetric procedure of Shinohara (8) was employed. In this method the color develops promptly with cysteine and over a prolonged period when homocysteine is present. In no case was there any lag in the production of the color.

B. Results

All computations are based on the tissue weights of the dead animals after the gut had been dissected, washed out, and returned to the carcass. These values are not offered as absolute, since the number of animals analyzed was small, in some instances only one individual. However, the figures obtained from the duplicate experimental rats are in good agreement and correspond to the figures given by Rose and Wood (2), or an increment of 3.6 mg. cystine per gram of tissues for rats receiving methionine supplements.

It may be seen that there is also close agreement between the values obtained for the two experimental rats *B* and *C* and the control of the same weight, in all respects except liver fat, which is absolutely and relatively higher in the former (Table 2). Liver fat is somewhat lower in *C* (5 per cent fat diet) than in *A* and *B* (30 per cent fat diet), demonstrating that although homocystine brings about a marked elevation of the fat content of the liver, the absolute height is to some extent conditioned by diet. Body fat is not increased by homocystine feed-

TABLE 2

FAT CONTENTS OF RATS (*A*, *B*, *C*) MAINTAINED FOR 65, 85, AND 98 DAYS ON A DIET DEVOID OF CHOLINE, METHIONINE, AND CYSTINE, SUPPLEMENTED WITH HOMOCYSTINE (Rats *D*, *E*, *F*, and *G* were controls.)

	A	*B*	*C*	*D*	*E*	*F*	*G*
Carcass-fat (gms.)	18.10	22.53	18.84	9.47	9.09	20.83	36.97
Carcass-fat, per cent fat-free carcass	13.1	16.3	13.7	10.0	8.9	16.1	16.2
Liver-fat (gms.)	1.84	1.71	1.21	0.26	0.29	0.30	0.52
Liver-fat, per cent fat-free liver	31.9	29.6	22.9	4.2	4.1	4.2	5.9
Total fat per cent total carcass	12.3	14.4	12.3	8.7	7.9	13.5	13.6

ing nor by the composition of the diet. It is striking that the oldest and heaviest control, *G*, contains the same percentage of fat as the younger, *F*, and as the experimental animals of the same age.

It has been shown by Womack and Rose (9) that only approximately one-sixth of the methionine requirement of the rat can be satisfied by cystine. Since this paper demonstrates that these amino acids

TABLE 3

TOTAL N_2, AMINO *N*, METHIONINE AND CYSTEINE (CYSTINE) CONTENTS OF RATS (*E*, *F*, AND *G*) MAINTAINED ON NORMAL DIETS AND (*B* AND *C*) DIETS DEVOID OF CHOLINE, METHIONINE, AND CYSTEINE, SUPPLEMENTED WITH HOMOCYSTINE

	B	*C*	*E*	*F*	*G*
Gain in tissue-wt. (gm.)	49.1	43.4	——	38.0	157.6
Total-nitrogen (mE.)	325.7	360.7	202.1	410.0	625.0
Amino nitrogen (mM.)	256.0	252.8	170.5	242.5	450.9
Total-methionine (mM.)	4.24	3.95	2.18	3.32	6.42
$\frac{\Delta \text{ methionine (mg.)}}{\Delta \text{ tissue (gm.)}}$	6.25	6.08	——	4.47	4.02
$\frac{\Delta \text{ methionine (mg.)}}{\Delta \text{ days}}$	3.11	3.07	——	8.91	7.36
Methionine, per cent tissue	0.38	0.36	0.27	0.32	0.35
Total cysteine (mM.)	3.76	3.76	2.52	3.74	6.37
$\frac{\Delta \text{ cysteine (mg.)}}{\Delta \text{ tissue (gm.)}}$	3.05	3.48	——	3.89	2.96
$\frac{\Delta \text{ cystine (mg.)}}{\Delta \text{ days}}$	1.52	1.74	——	7.81	5.42
Cysteine, per cent tissue	0.27	0.28	0.26	0.29	0.28
$\frac{\Delta \text{ S (mAt.)}}{\Delta \text{ tissue (gm.)}}$	0.067	0.067	——	0.062	0.051
$\frac{\Delta \text{ S (mAt.)}}{\Delta \text{ days}}$	0.033	0.035	——	0.094	0.124

are synthesized in approximately equimolar amounts, the other two-thirds of the methionine required must take part in separate reactions probably involving transmethylation. This high demand of the body for methyl groups may explain the findings of Mulford and Griffith (10) that a 26 per cent casein diet will not supply the cystine, methionine, and extra methyl needed to prevent choline deficiency in rats growing at the rate of theirs, whereas a 30 per cent casein diet is adequate. It is probable that slight, if any, methionine to cystine conversion occurs on this high casein intake.

Another striking observation which has emerged is the low percentage utilization of sulfur displayed by these animals in spite of the limited amount provided in the diet. Rat *C* received 36 mg. of homocystine daily, representing 0.27 mAt of sulfur. Its tissues gained 0.035 mAt of *S*, as methionine and cystine, amounting approximately to one-eighth of the sulfur ingested. It would be of interest to find how much sulfur was stored in other forms.

C. Conclusion

The synthesis of methionine and cystine from homocystine by rats in the absence of choline has been demonstrated.

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BOOK REVIEWS

Growth, 1944, **8**, 95-96.

(Darrell Haug Davis. The Earth and Man. New York: Macmillan, 1943. Pp. 675.)

REVIEWED BY FREDERICK S. HAMMETT

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An interest in growth implies, or rather it should imply, an interest in all factors whereby growth is conditioned. Knowledge of such is not attained by restricting oneself to learning more and more about less and less. It is attained by following the syncytial extensions whereby growth and other fields of scientific interest are connected. The scientist does this by natural inclination; the worker-in-science by prodding only.

It takes no great insight to allow that there are many protoplasmic bridges between growth and geography. Growth is from the land. And its expression is factored by the geography of the land from which it comes. Conversely, geography is of the land. And its expression is factored by the growth which that land sustains.

This growth-geography interdependence should be of as much, if not more, interest to the student of growth than to the student of geography. For growth is more subject to geographic factors than geography is to growth.

So this book of Davis' is a valuable addition to the library of any catholic minded student of growth. It is valuable not so much because it is a geography with a multitude of new pictures which will please children of all ages; as because it is a geography which deals with "the growth of geographic knowledge and mapping:—the development of the present objectives and fields of geographic study . . . the distribution of man" (population growth and change). ". . . the interaction between man and environment at different stages of human development; . . . the changes in environment resulting from operation of both natural and human agencies," and the "Limiting effects of the Environmental Factors" to the end that "understanding

of the limitations each imposes on the potentialities of an area for effective human use" may be envisaged.

This treatise on Human Geography then is an integrated exposition of the interdependence of land and man. And since man's primary activities are directed towards satisfying his basic need for food, clothing and shelter, the book of necessity deals copiously with land-utility and land-use for growth of the materials which supply these needs. It is thus a presentation from the geography side, of the growth-geography interdependence. Would that we had one equally as illuminating from the growth side.

Growth, 1944, **8**, 97.

(Robert Graves and Alan Hodge. The Reader Over Your Shoulder. New York: Macmillan, 1943. Pp. 446.)

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The book gives examples of the use and abuse of English and the principles of clear statement with examinations and fair copies. For anyone who wishes to present his data and his ideas thereon with precision, clarity and intelligibility, without ambiguity, confusion, or obscurity the volume will be a very present help in time of trouble. The following quotations give its tenor.

"To write English well, it is generally agreed, is not to imitate, but to evolve a style peculiarly suited to one's own temperament, environment and purposes." "We suggest that whenever anyone sits down to write he should imagine a crowd (sic) of his prospective readers (rather than a grammarian in cap and gown) looking over his shoulder. They will be asking such questions as: "What does that sentence mean?" "Why do you trouble to tell me that again?" "Must I really read this long, limping sentence?" "Haven't you got your ideas muddled here?" "There should be two main objects in ordinary prose writing: to convey a message and to include in it nothing that will distract the reader's attention or check his habitual pace of reading—he should feel that he is seated at ease in a taxi, not riding a temperamental horse through traffic." ". . . faults in English prose derive not so much from lack of knowledge, intelligence or art as from lack of thought, patience, or good will."

Good prose for modern conditions will be: "Cleared of encumbrances for quick reading: that is, without unnecessary ornament, irrelevancy, illogicality, ambiguity, repetition, circumlocution, obscurity of reference. Properly laid out: that is, each sentence a single step and each paragraph a complete stage in the argument . . . . with each idea in its right place in the sequence and none missing; with all connections properly made. Written in the first place for silent reading, but with consideration for euphony if read aloud."